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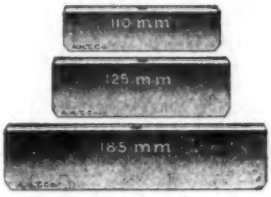

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OXIDATION-REDUCTION INDICATOR PATTERNS IN REGENERATION OF THE FORELEG AND TAIL OF TRITURUS TOROSUS LARVAE

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In various hydroids, *e.g., Corymorpha* and *Hydra,* reconstitution after removal of a part is largely or entirely by redifferentiation of tissues not directly involved in the injury and often at a considerable distance from it. In the planarian some new tissue which may be regarded as a blastema develops at the surface of injury and gives rise to head and posterior end but more or less extensive redifferentiation of other parts at various distances from the region of injury also occurs. In many cases almost the entire isolated piece undergoes reconstitution into some other part. In these organisms a marked increase in rate of oxidation and reduction of indicators appears at the injured surface almost immediately following removal of a part, *e.g.,* by section, and extends a variable short distance into the intact tissues. This is evidently an injury reaction. As reconstitution progresses, the region of redifferentiation also shows increased reactivity to the indicators, in many forms even before morphological differentiation is evident. *Corymorpha* is a particularly good example of such change in physiological condition preceding morphogenesis (Child and Watanabe, 1935; Watanabe, 1935). In the planarian there is also alteration of indicator pattern adjoining the surface of injury as an injury reaction and in other parts during the following reconstitution but the new tissue regenerating from the cut surface shows more activation than the redifferentiating parts (Child, 1948a).

The reconstitutional capacity of legs, tail, gill filaments and balancers in urodele amphibians has long been known. These and many other cases of reconstitution are commonly called regeneration because regeneration appears to be accomplished by development of a blastema on the surface of injury, apparently involving more or less dedifferentiation of cells with proliferation, growth and redifferentiation, usually into the part removed, and apparently with little or no alteration or redifferentiation of other parts. It has seemed of some interest to attempt to determine whether or to what extent alteration of oxidation-reduction indicator pattern is involved in a case of this sort.

Material and Methods

For leg regeneration *Triturus* larvae 12–20 mm. in length were used, in most cases larvae in which one or more toes were developing on the foreleg. Legs were

amputated at various levels. For tail regeneration larvae ranging from 10 to 16–18 mm. were used with different levels of amputation. The experimental larvae were fed every 2–3 days on pieces of enchytraeids. In stocks not sufficiently fed and with more than a single individual in a container legs were frequently bitten off, usually near the foot. These provided incidental material for stages of regeneration, though time of amputation was not known. These larval stages were used because the regeneration was rapid and because pigment development was not sufficient to obscure indicator patterns.

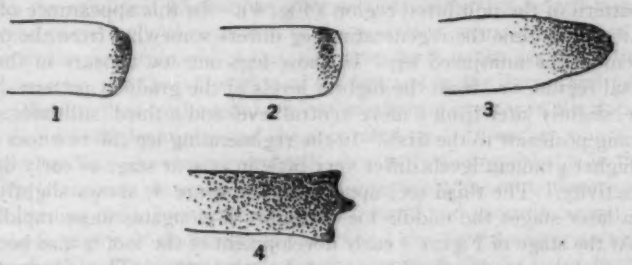
Intracellular formation and reduction of indophenol and intracellular oxidation of the dyes Janus green (diazine green) and methylene blue in solutions reduced by sodium hydrosulphite were used for intracellular oxidations. Following intracellular oxidation (primary oxidation) intracellular reduction of indophenol was obtained by addition of sodium hydrosulphite and of the dyes by addition of more hydrosulphite after primary oxidation. The indophenol reaction was used with very much lower concentrations of the reagents, para-aminodimethyl aniline (dimethyl paraphenylene diamine) and α-naphthol than in the original Nadi reaction. Use of the extremely low concentrations of the indophenol reagents has been described at length in other papers (*e.g.*, Child, 1948a; 1951). The intracellular reaction occurs in a very wide range of low concentrations of the reagents and with the very low concentrations it becomes an exceedingly sensitive indicator of certain differentials in physiological conditions and their changes in living material without appreciable injury but the most satisfactory concentrations for any particular material have been determined by trial. In the present cases, rather rapid reaction being desired, aniline 1/8000–1/16000, assuming that the sample is pure, and naphthol in much lower unknown concentration were used. Enough of the naphthol dissolves in water without heating and without addition of NaOH to give the reaction readily but amount of naphthol dissolving and actual concentrations used are unknown. Differential reduction of intracellular indophenol by sodium hydrosulphite and re-oxidation by oxygen was possible in living material. As a reducing agent sodium hydrosulphite is far superior to the other highly toxic reducing agents used in earlier studies of indicator pattern. Only very small amounts are required for reduction of indophenol or dyes; a small fraction of a milligram in one ml. of water will reduce intracellular indophenol or oxidized dyes in solution or within cells. Hydrosulphite is non-toxic in concentrations much higher than those required for reduction, except that long exposure without re-oxidation may inhibit or kill by lack of oxygen. For intracellular oxidation in reduced dye solution excess of hydrosulphite beyond that required to reduce the dye should be avoided. Excess of the reducing agent may prevent intracellular oxidation. Patterns of intracellular indophenol reaction and of dye oxidation in reduced solution and patterns of intracellular indophenol and dye reduction do not differ appreciably, *i.e.*, regions which oxidize more rapidly also reduce more rapidly than others until high intracellular concentrations of indicators retard or completely prevent reduction differentially.

Figures are necessarily diagrammatic attempts to indicate the differentials in indicator patterns following section and during the earlier stages of regeneration of foreleg and tail.

The kindness of Dr. V. C. Twitty in permitting use of material from his stocks is gratefully acknowledged.

Foreleg Regeneration

Almost at once after amputation, at least as soon as the indophenol reaction or primary intracellular oxidation of dyes became visible, color appeared at the tip of the leg stump earlier than in any other part of the body and gradually extended proximally in the stump for a short distance, usually less than a millimeter, in a decreasing gradient. This injury reaction involved not only the surface of the stump but also internal tissues including the bone which sometimes protruded from the stump. Also the anteroposterior and dorsiventral gradient pattern of the leg (Child, 1943b) usually became distinguishable in slightly more rapid color reaction anteriorly and dorsally in the stump. The injury pattern with anteroposterior differential is indicated in Figure 1. The dorsiventral differential is similar. Patterns of indophenol



Figures 1–4. Regeneration of the larval foreleg. Figures 1 and 2, injury reaction, as indicated by intracellular indophenol: Figure 1, early injury reaction, the anteroposterior pattern of the leg appears in the greater reactivity of the anterior side, uppermost in the figure; Figure 2, 1½–2 hours after amputation, intracellular reduction of indophenol at the extreme tip of the stump, without external oxygen decrease. Figure 3, twelve days after amputation just above knee, primary intracellular oxidation of Janus green to red diethyl safranin in solution reduced to colorless by sodium hydrosulphite, longitudinal and anteroposterior gradient patterns of the leg are evident in the regenerate and increased reactivity extends proximal to the level of amputation. Figure 4, sixteen days after amputation just below knee, indophenol reaction, three toes develop almost at the same time, the longitudinal gradient decreases proximally from the tips of the toes, the anteroposterior pattern from the anterior (upper) side of the figure.

reaction and primary intracellular oxidation of dyes are the same. Later more or less reduction of indophenol and dyes often occurred at the tip of the stump without external oxygen decrease and with the same anteroposterior and dorsiventral differential as the oxidase reaction (Fig. 2). It was noted earlier (Child, 1943b) that such secondary differential reduction often occurred, at least distally, in intact developing legs, gill filaments and balancers without external oxygen decrease and in oxidized dye solutions. Its occurrence suggests high oxidase activity. During the first 2–3 days following amputation the injury reaction appeared to decrease slightly in some cases. An injury reaction also occurred adjoining the cut surface of the distal amputated part of the leg with the same anteroposterior and dorsiventral pattern.

During the earlier stages of regeneration the indophenol reaction and primary dye oxidation in reduced solution were more rapid in the regenerating tissue than in more proximal levels of the leg and other regions of the animal. Anteroposterior and dorsiventral gradients were evident in the regenerating tissue, as well as in the more proximal intact parts and the increase in reactivity appeared to extend a short

distance into the intact part of the leg proximal to the level of amputation (Fig. 3). The reduction differentials following the primary oxidative reaction were similar until intracellular concentrations of indophenol or oxidized dyes became so high that reduction was differentially retarded or completely inhibited in the most susceptible regions in which the primary oxidative reaction was most rapid. Under these conditions more or less reversal of the color gradient pattern occurred.

Sixteen days after amputation approximately at the knee early stages of 3 toes were visible in some individuals and the exact boundary between the regenerated tissue and more proximal regions was becoming less distinct. The increased reactivity of the regenerating region was still evident and still appeared to extend somewhat proximal to the level of amputation. In the regenerated region the reactivity decreased proximally from the tips of the toes and continued without sharp boundary into the pattern of the uninjured region (Fig. 4). In this appearance of three toes at almost the same time the regenerating leg differs somewhat from the order of toe development in the uninjured leg. In those legs one toe appears in the dorsal or anterodorsal region, *i.e.*, from the highest levels of the gradient pattern of the leg, a second toe, slightly later from a more ventral level and a third, still later, also dorsal but becoming posterior to the first. In the regenerating leg the two toes developing from the higher gradient levels differ very little in rate or stage of early development and in reactivity. The third toe, uppermost in Figure 4, shows slightly less reactivity. In later stages the middle toe of Figure 4 elongates more rapidly than the others. At the stage of Figure 4 early development of the foot is also becoming evident in the slightly broader distal region of the regenerate. The anteroposterior and dorsiventral differentials in the pattern of the leg become evident progressively from the tips of the toes proximally through the regenerate into the proximal region of the leg. Progress of reduction is in the same direction until toxic effect retards or inhibits reduction distally, first of all in the toes. With rapid oxygen increase in the solution re-oxidation pattern is like the pattern of primary oxidation but with slight or slow oxygen increase is reversed. The superficial tissues of the leg were injured by the indicators earlier than the internal tissues, so that it was possible at a certain stage of toxic effect to observe more or less reversal of the original reduction gradient pattern superficially with persistence of the original pattern in the internal tissues. In later stages of leg regeneration the pattern gradually approaches that of the intact developing leg, as described elsewhere (Child, 1943b), consequently further consideration of the regeneration pattern is regarded as unnecessary.

Regeneration of the Tail

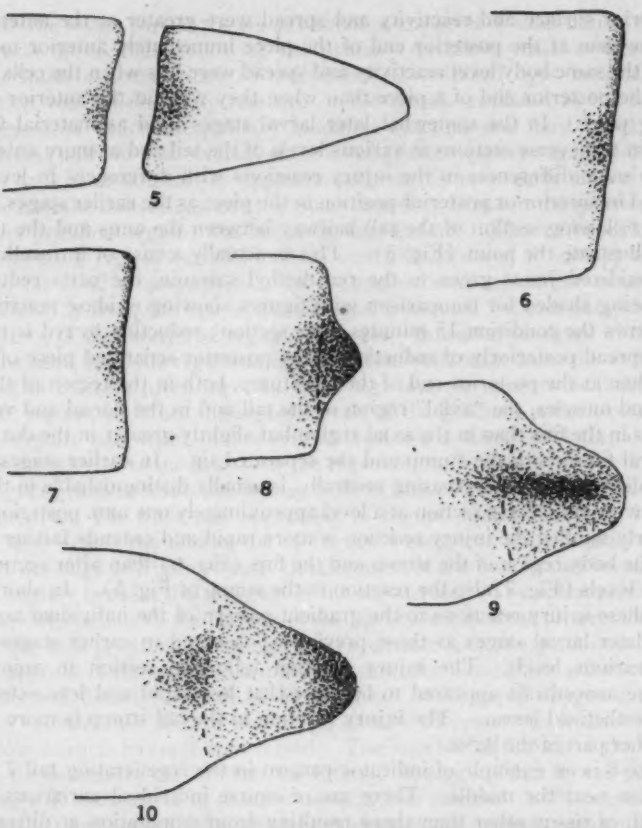
In an earlier study of indicator patterns in early amphibian development (Child, 1948b, pp. 95–99) it was noted that when larval stages earlier than those used in the present paper were completely separated into successive pieces by transverse section at different levels it was found that the reactivity, as indicated by intracellular indophenol, intracellular oxidation of dyes in reduced solution, and intracellular reduction of both indophenol and dyes by hydrosulphite adjoining both anterior and posterior cut surfaces, decreased from anterior to posterior levels of section of the body and tail and from dorsal to ventral at each level of section. In each piece the reaction was more rapid and the spread of the reaction from the cut surface was greater in the posterior direction from the anterior cut surface than in the anterior direction from

the posterior surface and reactivity and spread were greater at the anterior end of each piece than at the posterior end of the piece immediately anterior to it, *i.e.*, at virtually the same body level reactivity and spread were less when the cells concerned were at the posterior end of a piece than when they were at the anterior end of the following piece. In the somewhat later larval stages used as material for tail regeneration transverse sections at various levels of the tail and at more anterior levels show the same differences in the injury reactions with differences in level of body or tail and in anterior or posterior position in the piece as the earlier stages. A single example following section of the tail halfway between the anus and the tip is sufficient to illustrate the point (Fig. 5). This is actually a case of intracellular reduction of oxidized Janus green to the red diethyl safranin, the parts reducing most rapidly being shaded for comparison with figures showing oxidase reactivity. The figure shows the condition 15 minutes after section: reduction to red is more rapid and the spread posteriorly of reduction in the posterior separated piece of the tail is greater than at the posterior end of the tail stump, both in the region of the nervous system and muscles, the "axial" region of the tail and in the dorsal and ventral fins, much less in the fins than in the axial region but slightly greater in the dorsal than in the ventral fin in both the stump and the separated tip. In earlier stages of the reaction a slight gradient, decreasing ventrally, is usually distinguishable in the fins.

Following transverse section at a level approximately one mm. posterior to the tip of the early hindleg the injury reaction is more rapid and extends farther anteriorly in both the body region of the stump and the fins (Fig. 6) than after section at more posterior levels (Fig. 7, also the reaction in the stump of Fig. 5). In short, the relations of these injury reactions to the gradient pattern of the individual are the same in these later larval stages as those previously observed in earlier stages after section at various levels. The injury reaction following section in animals under chloretone anaesthesia appeared to be somewhat less rapid and less extensive than in unanaesthetized larvae. The injury reaction in the tail stump is more rapid than in any other part of the larva.

Figure 8 is an example of indicator pattern in the regenerating tail 7 days after amputation near the middle. There are of course individual variations in rate of outgrowth of tissue other than those resulting from amputation at different levels. The outgrowth is chiefly in the middle region in which the nervous system, spinal cord and muscles develop. Dorsally and ventrally the outgrowth decreases. At this stage the outgrowth shows merely a general gradient decreasing anteriorly without visible distinction of particular parts. It is noted as a point of some interest that the increased reactivity extends into tissues anterior to the level of section with a further decrease anteriorly of the gradient for a distance almost equal to the length of the regenerate; even in this region the reactivity is greater than in other parts of the larva. In general, rate of tail regeneration and to some degree the increase in reactivity in the regenerate and anterior to the level of section decrease as level of section becomes more posterior. Detailed description of such differences seems unnecessary.

In more advanced stages of regeneration individual differences in rates of outgrowth and in conditions in the tissues of the outgrowth appear. For example, in Figure 9, 17 days after amputation, the usual gradient, decreasing anteriorly, appears but the developing axial structures in the regenerate show much greater reactivity than the rest of the outgrowth, also with a gradient decreasing anteriorly.



FIGURES 5–10. Regeneration of the larval tail. Figure 5, injury reaction following transverse section at middle of tail, as indicated by intracellular reduction of Janus green, 1/30,000, to red diethyl safranin after staining by the oxidized dye; rate of reduction is higher than in any other part of the animal and its extent, both in the caudal axial region and in the fins, is greater posterior than anterior to the level of section. The indophenol reaction pattern is the same.

FIGURES 6 and 7 show the difference in rate and extent anteriorly of the injury reaction in caudal axial region and fins, as indicated by indophenol, after section at an anterior tail level (Fig. 6) and a level posterior to the middle of the tail (Fig. 7): reactivity is much greater and extends farther anteriorly in the caudal axial tissues than in the fins.

FIGURE 8. The indophenol reaction gradient 7 days after sections, with slight decrease anteriorly in the regenerate and further decrease in the caudal axial region anterior to the levels of section and to a lesser degree in the fins; reactivity is still greater than elsewhere in the animal.

FIGURE 9. Indophenol reaction 17 days after section; much more rapid reaction in the regenerating caudal axial region with slight decrease anteriorly, than in the regenerating fins dorsal and ventral to it: anterior to the level of section reactivity decreases anteriorly but extends farther than in earlier stages: reactivity in relation to regeneration is still greater than in any other part of the animal.

FIGURE 10. Indophenol reaction in a slightly more rapid regeneration than Figure 9, 16 days after section: after ½ hour the regenerating caudal axial region has undergone secondary

The high reactivity of this region is evidently associated with certain developmental stages of these structures. Dorsal and ventral to this region there is much less rapid reaction in the developing fins. In the axial part of the tail the increase in reactivity extends farther anterior to the level of section than in Figure 8, with decrease in the anterior direction. In another case (Fig. 10) with somewhat longer regenerate 16 days after amputation the axial structures in the regenerate reacted at first much like those in Figure 9 but secondarily reduced indophenol almost completely without external oxygen decrease, suggesting greater dehydrogenase activity than in the case of Figure 9. In the individual of Figure 10 the increased reactivity also extends a considerable distance into the tissues anterior to the level of section but without secondary reduction.

In still later stages of tail regeneration the difference between the regenerate and more anterior levels gradually decreases and disappears as rate of regeneration decreases. Greater reactivity with a gradient decreasing anteriorly, except when secondary reduction occurs, persists longer in the axial region of the tail than in the fins. Since nothing of particular interest as regards indicator patterns has been observed in these later stages it seems unnecessary to describe them at length.

Intracellular reduction of indophenol and dyes shows the same differentials in pattern until differential retardation or inhibition of reduction resulting from toxic effect of indicators, appearing earliest in regions originally of most rapid indicator oxidation, alters or may reverse more or less completely the gradient pattern. Reoxidation pattern is similar to the pattern of primary oxidation if oxygen increase following reduction is sufficiently rapid. With slow or slight oxygen increase it is the reverse of the primary oxidation pattern.

Discussion

The appearance of the anteroposterior and the dorsiventral differentials in the injury reaction on the foreleg stump is of interest as evidence that the change in condition of the cells concerned in this reaction depends to some degree on their condition preceding amputation. The increase in reactivity is somewhat greater in cells representing higher gradient levels in the intact leg than in those of lower gradient levels, consequently the gradient pattern is not obliterated in the injury reaction or in later regeneration. Either the differences in physiological condition associated with their original position in the pattern of the intact leg persist more or less completely during the increased reactivity and the probable greater or less dedifferentiation characteristic of the regenerating tissue, or else the pattern is induced in them by the differentials of the stump. The presence of the pattern in the injury reaction preceding actual regeneration perhaps indicates its persistence without induction. However, in the later regeneration and differentiation of the regenerated part its relation to the stump and the ingrowth of nerves doubtless plays a part.

The development of three toes almost at the same time in the regenerating leg, rather than in a definite sequence, as in development of the intact leg is probably to be regarded as a consequence of the higher level of metabolic activity in the regenerating

reduction without external oxygen decrease after a primary reaction: a gradient of increased reactivity, decreasing anteriorly, is present anterior to the level of section chiefly in the caudal axial tissues without evidence of reduction: reactivity in relation to regeneration is still greater than in other parts.

leg than in ordinary development which determines decrease in time differences in progress of morphogenesis.

Following transverse section in the tail the great difference in rate and intensity of the injury reaction in the post-anal extension of the neural, spinal and muscular structures, the "axial" region of the tail, and the dorsal and ventral fins is of interest as evidence of a great difference in physiological condition in the two regions. The reaction in the caudal axial region is not only much more rapid but extends much farther anteriorly from the cut surface than the very slight reaction immediately adjoining the cut surface in the fins. However, even in the slight reaction of the fins the dorsiventral gradient is usually distinguishable in the slightly more rapid reaction of the dorsal fin, and not infrequently a decrease in rate of reaction ventrally is visible in both fins.

It is also of particular interest in relation to the general significance of gradient pattern that the injury reaction is not only more rapid in both body region and fins of the posterior separated piece of the tail than in the anterior stump but also spreads farther posteriorly in the posterior piece than anteriorly in the stump. These differences in injury pattern at posterior and anterior cut surfaces are similar to those observed throughout the entire length of earlier larval stages, separated into successive pieces by a series of transverse sections at different levels (Child 1948b, Figs. 16 and 17). These differences are obviously associated with the anteroposterior gradient pattern of the larval body. On the two sides of the same level of section the injury oxidation is more rapid and spreads farther posteriorly down, than anteriorly up the gradient.

An essentially similar relation to gradient pattern appears in many other organisms, either as a feature of reconstitutional morphogenesis, or of functional physiological activities in certain organ systems of developing or mature organisms. In isolated pieces of Hydra and the planarian the differentials in reactivity to oxidation-reduction indicators in relation to body-level and to distal and proximal, or anterior and posterior cut ends of the pieces are like those in the larva of *Triturus* (Child, 1948a). Moreover, as regards bipolar forms of hydroids it has been known for many years that hydranths or partial hydranths, developing at the aboral ends of bipolar pieces of *Tubularia* stem within certain limits of length, are usually smaller, develop less rapidly, and are often less complete, than those developing from the oral end. Similar relations appear in bipolar forms of *Corymorpha*. Also in bipolar planarian heads the head developing from the posterior cut end is often smaller, develops less rapidly than the anterior head, and may show all degrees of differential structural inhibition. In both the hydroid and planarian bipolar forms the hydranth or head developing with establishment of a new gradient opposed in direction to the original gradient, involves greater changes in organization than does development at the oral or anterior end and may be more or less inhibited by the preëxisting gradient. However, in both hydroid and planarian pieces so short that the longitudinal gradient pattern is almost completely absent and consequently has little or no appreciable effect on development, apical regions or heads at both ends develop at about the same rate and under almost the same conditions as regards gradient pattern and are usually alike or almost alike.[1]

[1] As regards the bipolar forms of hydroids see, for example, Child (1907) and earlier literature cited and discussed there; Child and Watanabe (1935); Watanabe (1935); Child (1941; pp. 36–38, 313–321). For planarian bipolar forms see Rustia (1925); Child (1941; pp. 363–364).

The following examples of relations of functional physiological activities to gradient pattern are also of interest as indicating that morphogenetic and functional relations to gradient pattern are not fundamentally different. In the ctenophore plate row conduction of the impulse determining movement of the plates, following local mechanical or other stimulation at any level of the row, progresses much farther down than up the row. Usually it progresses down the gradient present in the row over the entire length of the row below the levels of stimulation but, unless the stimulation is extreme, only over a few plates up the gradient. The mammalian intestine possesses a longitudinal gradient pattern with high end at the pylorus, and, as Alvarez has demonstrated in a series of papers, conduction following a local stimulus at some level progresses much farther, often over the entire length down the gradient, and over a much shorter distance up the gradient. The vertebrate heart also gives evidence of somewhat similar relations (Child, 1941, pp. 327–330 and references cited there).

The much more rapid outgrowth in the caudal axial region than in the fins is what may be expected in view of the evidence of much more rapid metabolism in that region than in the fins. It is also of interest that the increase in reactivity in the axial region anterior to the level of section with a gradient decreasing anteriorly is characteristic, not only of the injury reaction but of regeneration (Figs. 8, 9 and 10). Evidently the change in physiological condition anterior to the surface of section is associated with the regenerative activity of the caudal axial region. In the fins there is also some increase in the spread anteriorly of slightly increased reactivity. It appears that even in regeneration of amphibian leg and tail which are commonly regarded as a regeneration from a blastema developing at the cut surface, the indicators give evidence of a change in physiological condition extending into tissues anterior to the level of amputation. This suggests the possibility that some degree of redifferentiation may be occurring there and that this case is perhaps not fundamentally different from reconstitution in various invertebrates, *e.g.*, planarians, which is only in part a real regeneration.

In earlier indicator studies on amphibian development (Child, 1943a, 1943b) it has been stated that the re-oxidation gradient, following intracellular reduction, is opposite in direction to the gradients of primary oxidation and reduction, *i.e.*, that regions which primarily oxidize and reduce the indicators most rapidly re-oxidize them after reduction least rapidly and that rates of re-oxidation increase as rates of primary oxidation and reduction decrease. In further work with indicators this has been found to be true when external oxygen increase is relatively slow and slight. However, when it is sufficiently rapid and great the re-oxidation pattern is the same as that of primary oxidation. Larval amphibian appendages frequently reduce indophenol and dyes secondarily, *i.e.*, after first oxidizing them, and without external oxygen decrease, suggesting high dehydrogenase activity. In re-oxidation apparently oxygen available, rather than enzyme activity, may determine the re-oxidation differential if oxygen increase is slow or slight because it becomes sufficient to permit intracellular re-oxidation earlier in regions of lower, than in those of higher oxygen uptake. With rapid and great external oxygen increase the differentials in enzyme activity become the factor determining the re-oxidation pattern. In short, two opposed re-oxidation patterns are possible, not only in amphibian development but in other organisms, one determined by external, the other by internal factors.

Summary

1. Amputation of foreleg and tail at various levels is followed almost immediately by increased intracellular indophenol reaction and oxidation of dyes in reduced solution.

2. In the leg the injury reaction and the later regenerative outgrowth show the anteroposterior and dorsiventral patterns of the leg.

3. Development of the first three toes at almost the same time in the course of regeneration of the foot, rather than in the sequence characteristic of the development of the intact leg probably results from the increased metabolism in the regenerating, as compared with the intact leg.

4. In tail regeneration the injury reaction is more rapid and extends much farther anterior to the surface of amputation in the caudal axial region than in dorsal and ventral fins. Also the later regenerative outgrowth is much more rapid in and near the caudal axial region than in the fins. The reactivity gradient decreases anteriorly.

5. The increase in reactivity anterior to the level of tail amputation is present not only during the injury reaction but also during the following regeneration. Its persistence suggests that considerable physiological change may occur in this region, as well as in the regenerative outgrowth.

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AN ANNUAL HISTORY OF A POPULATION OF NEREIS DIVERSICOLOR O. F. MÜLLER

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Nereis diversicolor O. F. Müller is probably one of the most numerous mud-flat animals in Europe. Its tolerance of widely different environmental conditions, especially its resistance to varying salinity, is well known but not fully understood, and although a certain amount of laboratory work has been performed on this animal, it has been little studied in nature. As the rate of growth of these worms, length of life and related phenomena have hitherto been unknown, a study of the history of a single population is of interest.

Observations on a population of *Nereis diversicolor* were made at Chalkwell, Essex, on the north bank of the Thames estuary in England, during 1948–1949. Population densities were determined by taking five or six 0.25 m.^{2} samples, in each instance dug to a depth of about 40 cm. Monthly samples were taken within a fairly limited area of shore about 1000 m.^{2} in area at the upper limit of the mud-flat zone where there was an average exposure of 7 hours at each tide. A frame was used with sides 30 by 83.3 cm. enclosing an area of approximately 0.25 m.^{2} The length of the shorter side was chosen in relation to the breadth of the spade used to dig out the samples. Usually five samples were taken at random within the chosen area, which was small enough to eliminate any possible variations in numbers due to differences in tidal level, major substratum differences, or other factors, but large enough to preclude the possibility of any reduction in numbers due to over-sampling on successive occasions. The muddy sand was friable enough to make hand sorting relatively easy, and it was found that this method was as accurate as sieving. When worms were found in a fragmented condition, heads only were counted.

Worms were measured after narcotization with 10% magnesium sulphate on a wet glass sheet overlying squared paper. Although very contractile, measurement of length (excluding head and anal appendages) could be made with an accuracy of $\pm$ 0.5 cm. with worms of total length 10 cm. The measured length is probably rather greater than the real length owing to the action of the narcotic. The results have been arranged in size groups with extremes differing by 2.0 cm. Where worms were of such a length that they might have been referred to either of two adjacent size-groups, the lower group was consistently preferred. Samples taken for assessing the mean length were obtained by random digging.

I am indebted to Dr. G. E. Newell for many suggestions, and to Dr. C. Barker Jørgensen for reading the typescript.

Population Density

The monthly variation in numbers of worms per square metre (Table I) is shown graphically in Figure 1. The population density during the winter was fairly low,

but approximated to the same value (about 55/m.²) from January until March. This number represented the effective breeding population density, which contained less than 10 per cent of males (Dales, 1950). Although spawning took place in this population over a period of not more than about two weeks towards the end of February, the young worms resulting from successful fertilizations at this time did not reach a length sufficiently great to be recognised by the sampling method employed until the following June (Fig. 3). From March onwards there was a slight but significant fall in the number of worms per unit area, and this was due to the spawned,

TABLE I

Monthly variation of population density

Month	Number of worms in each 0.25 m.³ sample							Mean no./0.25 m.³	Mean no./m.³
1948									
October (29:10:48)	37	32						34.5	130.0
November (25:11:48)	25	38	39	16	19			27.4	109.6
December (22:12:48)	19	30	16	25				22.5	90.0
1949									
January (23:1:49)	14	8	12	13	18			13.0	52.0
February (24:2:49)	17	8	23	13	12			14.6	58.4
March (23:3:49)	11	17	20	10	11			13.8	55.2
April (21:4:49)	4	4	7	20	23	15	7	11.4	45.6
May (27:5:49)	5	15	2	10	11			8.8	35.2
June (22:6:49)	32	6	43	27	23			26.2	104.8
July (20:7:49)	23	66	70	56	79			58.8	235.2
August (19:8:49)	110	55	86	52	48			70.2	280.8
September (16:9:49)	48	76	74	43	68			61.8	247.2
October (24:10:49)	65	34	54	43	29			35.0	140.0

and later some of the unspawned individuals having died. Most of the unspawned females die during the summer, although some undoubtedly survive until the following winter, and although this is obscured by the increase in number of small worms in each sample in Figure 1, it is shown by the histograms (Fig. 3) for these months. By inspection of the data, the percentage of the total adult (1948) generation may be estimated at about 50 per cent in June, 22 per cent in July, and only 5 per cent in August. Unfortunately, a more accurate separation of these two components (the adult and juvenile parts of the population) was found to be impossible by the graphical arithmetic probability method. From June until August the density of the popu-

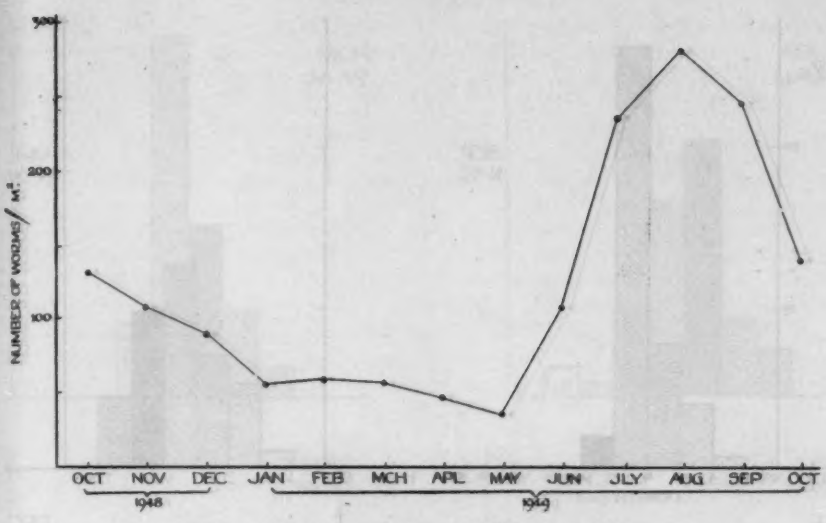

FIGURE 1. Monthly variation in density of population.

lation rose rapidly as the young worms became long enough to be seen in the sand. The population density reached a maximum of about 280/m.² at the end of August, after which the numbers decreased to approximately the same winter density as before. This fall in density was probably due to a thinning out of young worms, although a certain amount of emigration may also have occurred, while a small part was due to the elimination of the remnants of the previous generation.

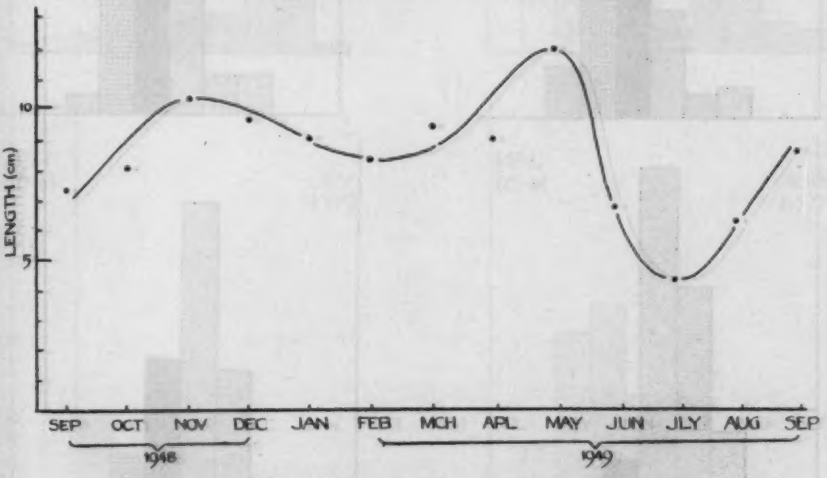

FIGURE 2. Monthly variation of mean length.

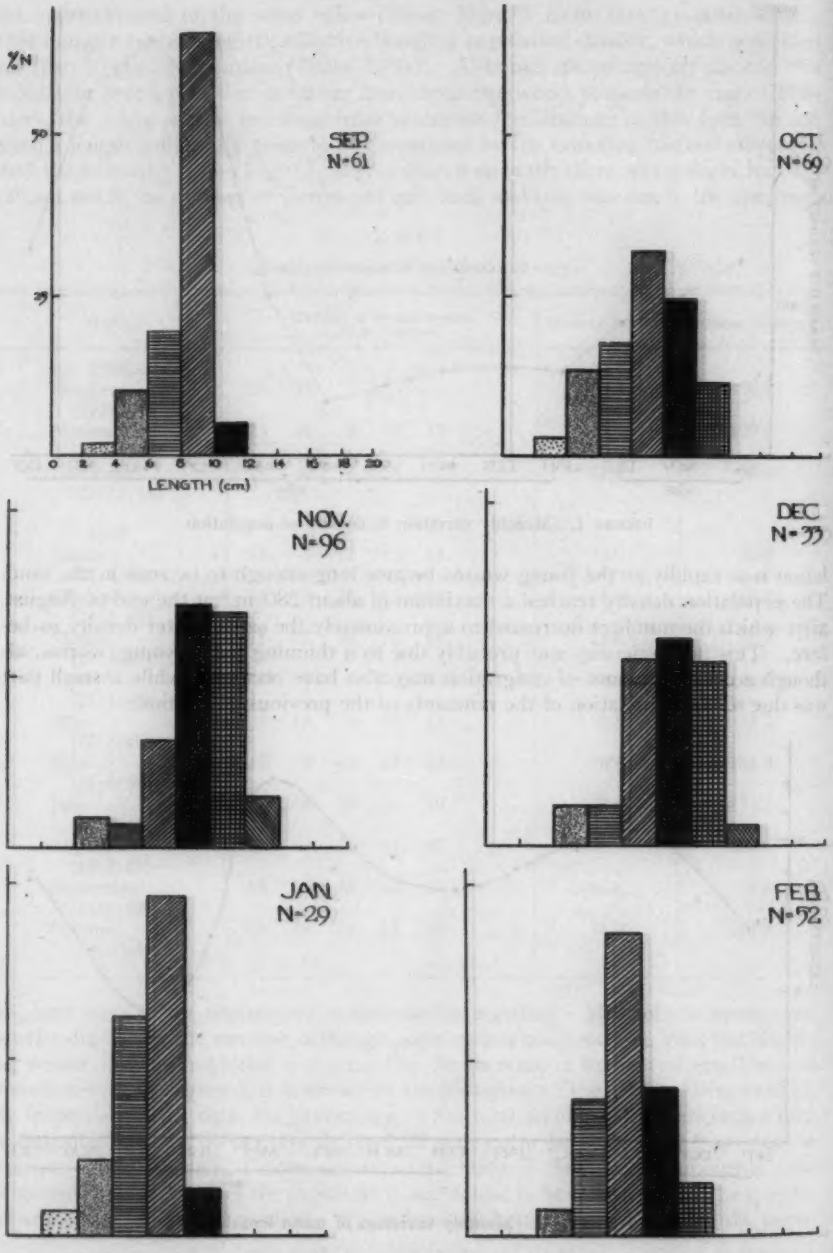

FIGURE 3. Histograms illustrating the monthly size distribution throughout the year.

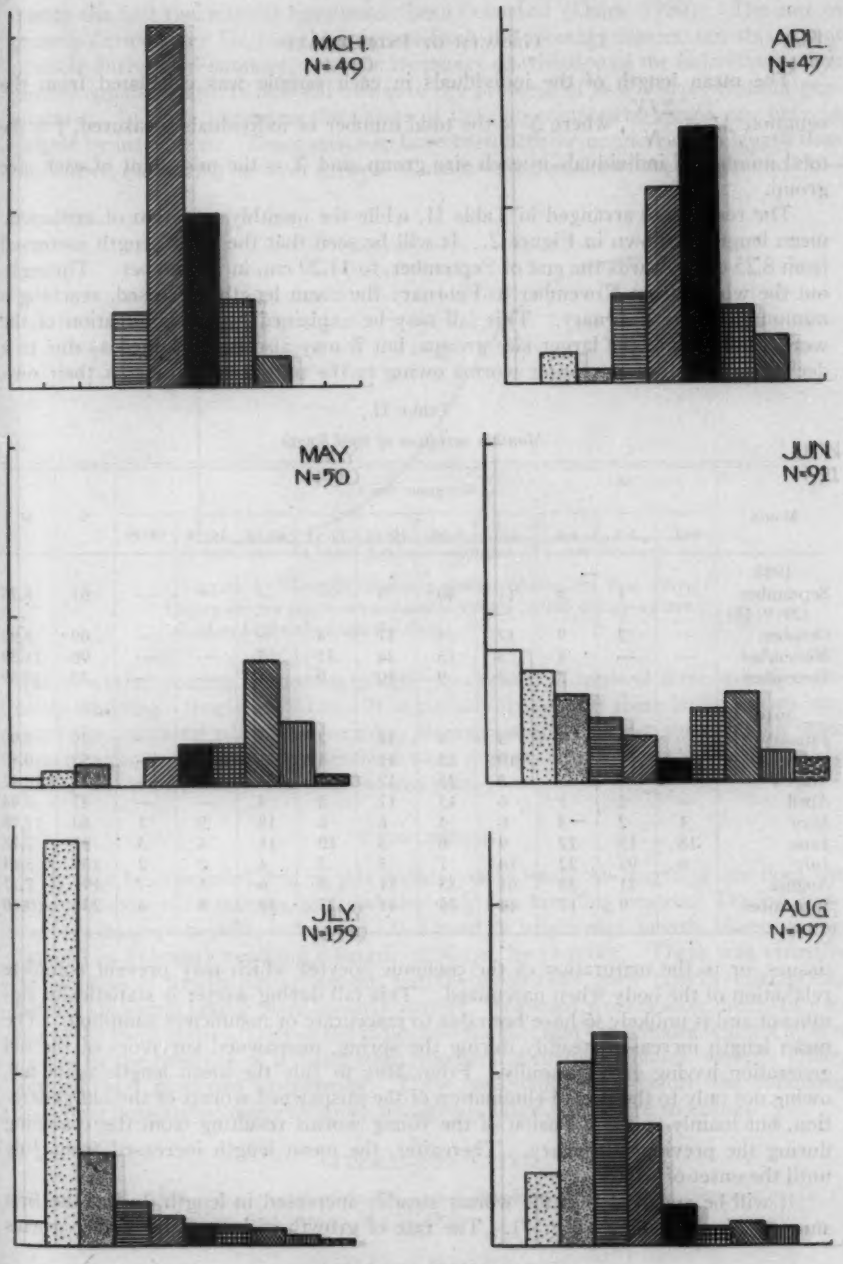

FIGURE 3.—*Continued.*

Growth of Individuals

The mean length of the individuals in each sample was calculated from the equation $M = \frac{\Sigma fX}{N}$, where N = the total number of individuals measured, f = the total number of individuals in each size group, and X = the mid-point of each size group.

The results are arranged in Table II, while the monthly variation of arithmetic mean length is shown in Figure 2. It will be seen that the mean length increased from 8.25 cm. towards the end of September, to 11.29 cm. in November. Throughout the winter from November to February the mean length decreased, reaching a minimum in late February. This fall may be explained by the elimination of the worms falling into the larger size groups, but it may also be explained as due to a decline in length of the larger worms owing to the partial resorption of their own

Table II

Monthly variation of total length

Month	Size-group (cm.)										N	M
	0–2	2–4	4–6	6–8	8–10	10–12	12–14	14–16	16–18	18–20		
1948												
September (29:9:48)	—	1	6	11	40	3	—	—	—	—	61	8.25
October	—	2	9	12	21	17	8	—	—	—	69	8.92
November	—	—	4	3	15	34	33	7	—	—	96	11.29
December	—	—	2	2	9	10	9	1	—	—	33	10.52
1949												
January	—	—	1	3	9	14	2	—	—	—	29	9.99
February	—	—	2	10	25	11	4	—	—	—	52	9.19
March	—	—	—	5	25	12	5	2	—	—	49	10.42
April	—	2	1	6	13	17	5	3	—	—	47	9.94
May	1	2	3	0	4	6	6	18	9	1	50	12.98
June	18	15	12	9	6	3	10	11	4	3	91	7.78
July	6	95	22	10	7	5	5	4	3	2	159	5.44
August	—	21	53	61	35	11	5	6	5	—	197	7.27
September	—	9	17	46	76	41	27	12	8	3	239	9.60

tissues, or to the maturation of the coelomic oocytes which may prevent complete relaxation of the body when narcotized. This fall during winter is statistically significant and is unlikely to have been due to inaccurate or insufficient sampling. The mean length increased steadily during the spring, unspawned survivors of the old generation having grown rapidly. From May to July the mean length again fell, owing not only to the partial elimination of the unspawned worms of the old generation, but mainly to the inclusion of the young worms resulting from the spawning during the previous February. Thereafter, the mean length increased from July until the onset of winter.

It will be apparent that the worms steadily increased in length during the first summer after hatching (Fig. 4). The rate of growth of larvae and young worms

during the first two months has already been described (Dales, 1950). The rate of growth during later life may be inferred from the monthly means, but this is not possible during mid-summer, since the frequency distribution of the individuals when plotted against length is bimodal owing to the presence of two sharply distinct generations. In these instances, the values of the two component means can only be judged by inspection. There seems to have been little or no increase in length during winter, but during summer growth of unspawned females of the old generation—

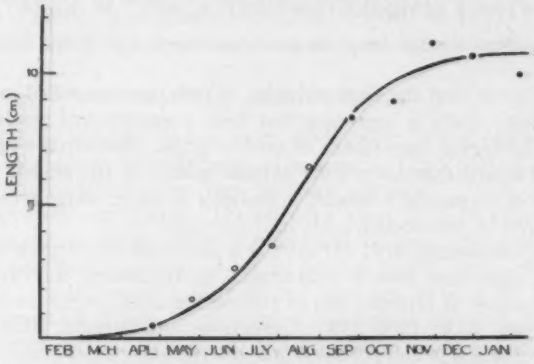

FIGURE 4. Growth of young worms during the first year. Empty circles represent estimated values; filled circles values calculated directly from the data.

that is, worms nearing 18 months in age—was resumed, some of these worms eventually attaining a length of 20 cm. It is unlikely that any of these latter worms survived the following winter. However, some females which for some reason were slow in maturing, and were undersized when a year old may possibly have lived to spawn (for the first time) in their second year.

CONCLUSIONS

It may be concluded that in this population at least, the length of life does not normally exceed 18 months, and includes only one breeding season. During summer worms grew rapidly, increasing 1.0–2.0 cm. in length each month, young worms hatched in February reaching a length of 10 cm. by October. There was virtually no increase in length during winter, but the germ cells grew rapidly during this period, spawning taking place in early spring. Spawned worms soon died, but owing to the comparative rarity of the males and the fact that the females will not spawn except in the presence of a male, large numbers of mature females were, in effect, surplus, and remained unspawned. These eventually died before the following spawning season, but many attained a relatively large size before so doing.

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EFFECT OF X-RADIATION ON THE DESOXYRIBONUCLEIC ACID AND ON THE SIZE OF GRASSHOPPER EMBRYONIC NUCLEI [1, 2]

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It is now believed that the vital activities of cells are controlled to a great extent by nucleoproteins. Only a beginning has been made toward attaining an understanding of the biological importance of nucleic acids. Research methods which involve the use of agents that disturb the normal balance of the nucleic acids are being utilized, and by these means it should be possible to reach some definite conclusions on the role played by the nucleic acids in living cells.

Since desoxyribonucleic acid (DNA) is a universal constituent of chromatin, it probably has a significant part in inheritance, in regulating cell division, and perhaps in the formation of proteins and of ribonucleic acids of the nucleolus and cytoplasm (Caspersson, 1936, 1940, 1941; Caspersson and Schultz, 1938, 1940). Since x-rays produce mutations and chromosomal aberrations, observations on their effect on the DNA content of the cells are of considerable importance. We had previously noted in this laboratory that irradiated nuclei seem to stain less intensively with the Feulgen reagent than non-irradiated nuclei. The purpose of the present study was to determine by photometric methods, which make possible an accurate quantitative cytochemical study of the nucleoproteins, whether there is an actual change in the quantity of DNA after irradiation. Examination of our photometric data sheets disclosed that the diameters of irradiated nuclei were usually larger than those of control nuclei. This fact, along with an apparent increase in size observed in irradiated neuroblasts in living culture preparations, led to a study of the effect of radiation on nuclear size.

Methyl green stain and the Feulgen reaction were used for the DNA determinations. With these two stains it is possible to analyze for two different properties of the nucleic acid. The Feulgen is specific for the DNA desoxypentose content and the methyl green probably indicates the presence of DNA in the normal state of polymerization, commonly called the "highly polymerized state." Most workers now agree that, when the nuclear reaction takes place under properly controlled conditions, the Feulgen method gives an accurate relative estimate of the DNA in nuclei (Hillary, 1940; Baker, 1942; Stowell, 1945, 1946; Di Stefano, 1948). Ultraviolet absorption studies of Wyckoff, Ebeling and Ter Louw (1932) identify the Feulgen-positive material in the nucleus with DNA. Also, splenic and pancreatic desoxy-

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[2] For work reported in this paper the authors were awarded the research prize of the Association of Southeastern Biologists at the 1951 annual meeting in Tuscaloosa, Alabama.

[3] This work was begun at the Department of Zoology and Entomology, University of Tennessee.

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ribonucleases remove the Feulgen-positive bands (presumed to contain large amounts of DNA) from the salivary gland chromosomes of Drosophila (Catcheside and Holmes, 1947). Although there is evidence that the reaction does not indicate the total amount of DNA present (Caspersson, 1932; Ely and Ross, 1949), many workers have shown it is valid to use the Feulgen reaction with the photometric system to determine the relative amounts of DNA in nuclei (Ris and Mirsky, 1949; Lumb, 1950; Leuchtenberger, 1950; Swift, 1950). The specificity of methyl green for polymerized DNA was demonstrated by Pollister and Leuchtenberger (1949), Leuchtenberger (1950), and Kurnick (1950a, 1950b, 1950c). Leuchtenberger found Feulgen and methyl green extinction ratios to be equal in normal nuclei.

Material and Methods

The present cytochemical studies were made with 14-day-old embryos of the grasshopper, *Chortophaga viridifasciata* (De Geer). The control and irradiated embryos for each experiment were from the same egg case. Doses of 4000, 10,000, and 12,500 r of x-rays were given. The embryos were fixed 10 hours after irradiation, since the maximum visible difference between the irradiated and control tissue, along with the maximum swelling of the irradiated nuclei, was observable at this time (Figs. 1–3). Both control and irradiated eggs were kept at 38° C. between the time of irradiation and fixation. Treatment of the control and x-rayed embryos was identical throughout each experiment except for the irradiation, thereby ruling out any disparity of the results from other causes and making valid a comparison of the cells of the irradiated and control embryos.

Since many cytologists (Koller, 1947; Darlington and Mather, 1949; Serra, 1949; etc.) believe that the content of DNA per nucleus varies with the different stages of mitosis, all photometric measurements were made on cells in the mitotic stages in which the chromatin is most widely dispersed, namely, late telophase, interphase, and early prophase. All three of these stages were used because it was difficult to distinguish them from one another in material which had been treated by immersion in approximately 20 per cent of the isotonic salt concentration to make the chromatin more uniformly distributed throughout the nuclei.

Two different x-ray units were used in these studies. The first experiments were done with a Coolidge Universal x-ray tube, operating at 90 kilovolts and 5 milliamperes. During exposure the material was 18.4 cm. from the center of the target, and the long wave length x-rays were removed by a 0.3 mm. aluminum filter. The intensity was about 250 r per minute. Later, when the previous experiments were repeated and additional studies made, irradiation was carried out with a General Electric Maximar 250 kv unit operating at 250 kilovolts and 15 milliamperes. An aluminum filter of 0.5 mm. was added to the 3 mm. aluminum inherent filtration of the machine. When the material was 54 cm. from the center of the target, the intensity was about 250 r per minute. Monitoring with a Victoreen thimble chamber preceded each x-ray treatment.

All material to be sectioned was fixed with Carothers fluid.[5] The fixation time

[5] 75 cc. of a saturated aqueous solution of picric acid; 15 cc. of formalin, C. P.; 10 cc. of glacial acetic acid; 1 gm. urea crystals. Warm slightly and stir thoroughly as the urea is added. Add 4 drops of a 50 per cent aqueous solution of chromic acid to 5 cc. of the above solution just before using.

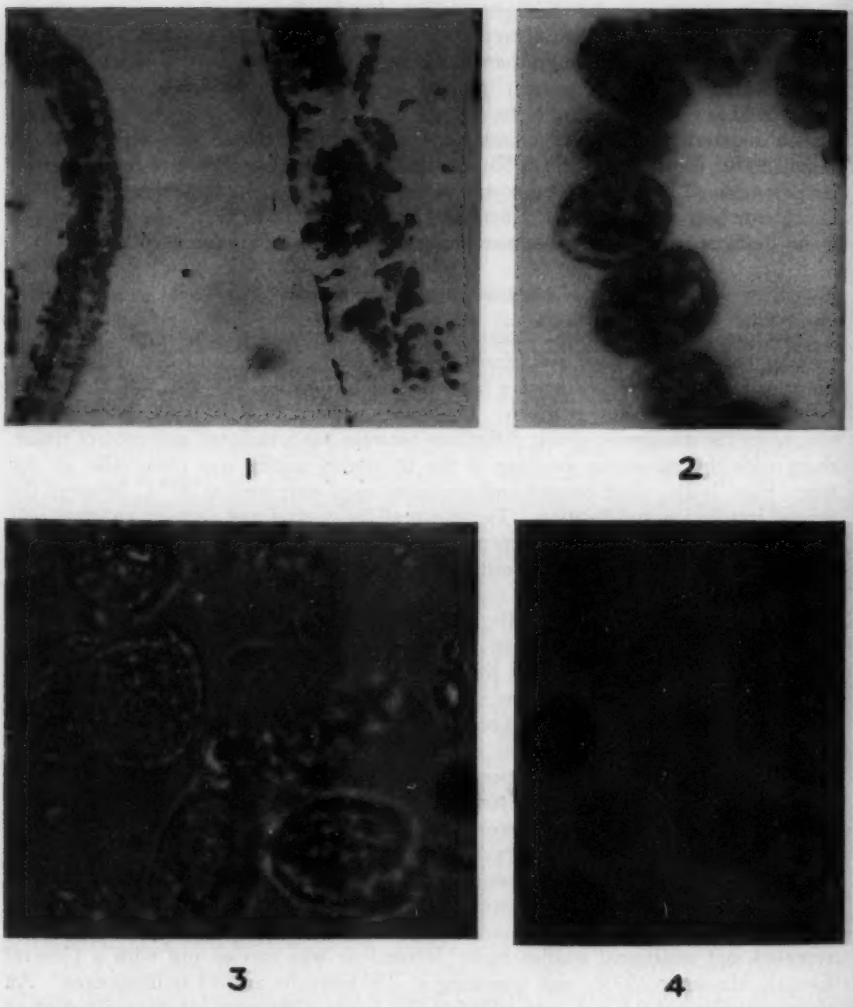

FIGURE 1. Note the difference in Feulgen stainability between the irradiated embryo (4000 r) on the left and the non-irradiated embryo on the right. Fixed with Carothers fluid 10 hours after treatment. 100 ×.

FIGURE 2. Heterogeneous un-irradiated nuclei. Carothers fixation. Feulgen stained. 900 ×.

FIGURE 3. Heterogeneous irradiated nuclei. Fixed with 4 per cent neutral formalin, stained with methyl green. Note the enlarged nuclei. 900 ×.

FIGURE 4. Un-irradiated nuclei pre-treated with a hypotonic solution that made the chromatin more uniformly distributed. Fixed in 4 per cent neutral formalin, stained with methyl green. 900 ×.

of the control and treated embryos for each experiment was the same, varying from 20 to 24 hours for the different experiments. The embryos were washed in distilled water for one hour and then prepared for sectioning by dehydrating with ethanol. The picric acid of the fixative was removed in a 70 per cent ethanol solution saturated with lithium carbonate. After dehydration was complete, the material was cleared in aniline oil and chloroform and embedded in Tissuemat. The control embryo and treated embryo were embedded side by side in the paraffin block and sectioned simultaneously. Sections were cut at 16 microns, then decerated and hydrated. A hydrolysis curve for each time was run in normal HCl (58° C.) for 10, 20, 30, and 40 minutes. The optimum Feulgen staining reaction, which was used for photometric determinations, was obtained after 20-minute hydrolysis. The Feulgen stain was prepared as specified by Coleman (1938). With the exception of the staining time, 3 hours for these sections, the staining schedule of Darlington and LaCour (1947) was followed.

Embryos dissected in a hypotonic solution and then fixed in acetic-alcohol or neutral formalin have nuclei which show a more uniform distribution of chromatin than cells dissected in an isotonic solution (Figs. 2, 3, 4). Variations in the absorption measurements of a nucleus were reduced by making the chromatin more uniformly distributed. Smears of the more uniform-appearing nuclei were stained with Feulgen and methyl green in the following manner. Embryos were removed from the eggs and dissected in a hypotonic culture medium and left in the solution for 10 minutes. Cells that were to be stained with the Feulgen reagent were fixed in Carnoy fluid (3:1) for 30 minutes. After hydrolysis for 10 minutes in normal HCl at 60° C., the embryos were rinsed in distilled water, stained with leucobasic fuchsin for 15 minutes, softened for smearing by treating with 45 per cent acetic acid for 1 minute, and then mounted and smeared in a Karo-formalin solution (1 part Karo, 1 part formalin, 3 parts water). The preparation and methyl green staining procedure described by Kurnick (1950a) was followed, with the exceptions that the embryos were dissected in a hypotonic culture medium before fixation and that the staining was done at 17° C. Cells treated in the same way except for staining were used to determine the blank, *i.e.*, the absorption of unstained nuclei. Correction for nonspecific light loss was not made after it was shown that unstained nuclei are practically invisible and transmit from 93 to 96 per cent of the light.

Measurements of nuclear diameters were made with an oil immersion objective (90 ×) and a 10 × ocular which contained a calibrated micrometer disk. Since all the nuclei were not exactly spherical, four different diameters of each nucleus were read and averaged. The mean of ten such averages, all on nuclei from the same embryo, was obtained for each dose of x-rays and for the corresponding controls. Over 300 nuclei were measured. An example of the data is shown in Table I.

The photometric apparatus used is similar to that described by Swift (1950) with the micro-ammeter replaced by an amplifier and Brown recorder. The recorder was originally installed to facilitate some tests of the apparatus, and since it offered the advantage of an automatic permanent record of the photoelectric current, it was not removed from the system. The DNA stained with the Feulgen reagent was measured by the absorption of green light isolated from a Spencer microscope lamp by means of Corning Glass filters No. 3484, No. 4010, and No. 4303. The DNA stained with methyl green was measured in terms of the absorption of red light iso-

lated by Corning Glass filter No. 2408. Optics were an A. O. 50 × reflecting objective and a Zeiss 10 × ocular.

DNA content is expressed in relative instead of absolute units, because several assumptions are made with photometric microscopic measurements, namely, that the nuclei are perfect spheres, and that the material is homogeneously distributed throughout the nucleus. With Feulgen-stained material variations in fixation and staining procedure may produce variations in the intensity of the reaction. Also, potentially stainable material may be lost during hydrolysis. In view of these as-

TABLE I

Data showing measurements of diameters of control and irradiated (4000 r) nuclei. Sections fixed with Carothers fluid, hydrolyzed 20 minutes, and Feulgen-stained

Embryo	Nuclei	Diameter measurements				Average	Mean
		1	2	3	4		
Irradiated (4000 r)	1	10.5	10.0	11.0	10.5	10.50	
	2	10.5	10.0	8.5	8.0	9.25	
	3	9.0	9.0	8.5	8.0	8.62	
	4	8.5	8.5	7.5	9.0	8.37	
	5	10.5	10.0	11.0	11.0	10.62	
	6	8.0	8.5	8.5	8.0	8.25	
	7	8.5	10.5	10.0	10.5	9.87	
	8	9.0	10.0	9.0	11.0	9.75	
	9	8.0	9.0	8.0	9.0	8.50	
	10	9.0	8.5	8.5	8.5	8.62	
							9.24
Control	1	8.5	10.0	10.0	10.5	9.75	
	2	7.5	8.0	8.5	7.5	7.87	
	3	7.5	8.0	8.5	7.5	7.87	
	4	6.5	7.5	7.5	7.5	7.25	
	5	8.5	8.0	6.0	7.5	7.50	
	6	8.0	8.5	8.0	8.0	8.12	
	7	9.0	9.0	8.0	9.0	8.75	
	8	8.5	8.0	10.0	9.0	8.87	
	9	9.0	8.5	9.0	10.0	9.12	
	10	9.0	8.5	9.0	10.5	9.25	
							8.43

sumptions and possible sources of error, it is not feasible to determine absolute amounts of DNA per nucleus. All values of DNA reported are, therefore, in arbitrary units.

A brief explanation of the photometric routine will aid in understanding the tables. A nucleus which is to be measured is centered in the microscope field and brought into sharp focus. The photometric apparatus is adjusted over the ocular, and an enlarged image of the nucleus is observed through a magnifying lens placed immediately above the field diaphragm of the photometer. Measurements of the nuclear diameter are made, after the nucleus has been made concentric with the micrometer disk in the image plane. An aperture of 1, 2, or 3 millimeter diameter is

inserted in the image plane, so that only a central core of the nucleus is visible. The magnifier is then swung out, and the phototube is moved into place over the aperture. Light from the central core of the nucleus now falls on the photosensitive cathode, and a reading is made with the Brown recorder. Immediately the slide is moved

TABLE II

Samples of raw data, showing the calculations made in obtaining the arbitrary units given in subsequent tables

Nuclear diameter* (μ)	I_n	I_0	I_0/I_n (1/Transmission)	Extinction (log 1/T)	1/F	EC² (1/F)
Nuclei irradiated 4000 r. Carothers fixation. Feulgen sections.						
9.2	59.1	93.2	1.577			
	59.6	92.7	1.555			
	59.6	92.4	1.550			
	60.7	91.7	1.511			
	58.8	92.4	1.571			
			1.553	0.19117	6.476	2.78
9.0	53.7	91.8	1.709			
	52.8	93.1	1.763			
	53.3	91.1	1.709			
	54.8	91.6	1.671			
	52.5	90.6	1.726			
			1.716	0.23452	6.115	3.23
Non-irradiated (control) nuclei. Carothers fixation. Feulgen sections.						
6.5	40.8	91.8	2.250			
	40.4	90.9	2.250			
	40.5	92.4	2.281			
	40.4	90.7	2.245			
	40.2	90.3	2.246			
			2.254	0.35295	3.29	2.61
6.2	30.1	86.6	2.877			
	29.1	87.1	2.993			
	29.0	87.6	3.021			
	29.8	87.2	2.926			
	30.6	87.4	2.856			
			2.935	0.46761	2.99	3.15

* An average of five measurements.

and a clear area of the slide is measured in a similar manner. Two readings, the first through the nucleus and the second through the background, are made for each determination. The intensity of the light passing through the clear area (I_0) divided by the intensity of the light transmitted by the nucleus (I_n) gives the reciprocal

of the transmission ($1/T$). Transmission data are then converted into extinction values (E):

$$E = \text{Log}_{10}\, 1/T.$$

This extinction value represents the light transmission for the central core of the nucleus and not through the entire nucleus. A value which gives the relationship of the area measured to the entire volume of the cell is $C^2\, 1/F$.

$$1/F = \frac{R^3}{R^3 - (R^2 - C^2)^{3/2}}$$

C is the radius of the cylinder or the radius of the aperture used to make the reading. R is the radius of the spherical nucleus. Then $EC^2 1/F$ = extinction value for the total volume of the nucleus (Table II). Further details of the computations and corrections for photometric measurements are discussed by Swift (1950), Pollister and Ris (1947), and Di Stefano (1948).

Statistical Treatment

To ascertain whether a significant difference existed between the mean diameters of irradiated and control nuclei, a test devised by Mosteller (1948) was used. The 2.0 per cent level was accepted as significant.

A two-way analysis of variance table was used to determine the difference in DNA (Feulgen and methyl green) content of irradiated and control nuclei. Unless stated otherwise, the 5 per cent probability level was accepted as the significant value.

Results

Nuclear diameter. When studying a series of Feulgen-stained nuclei of any one type, the fact becomes apparent that smaller nuclei are more darkly stained than larger nuclei. This condition was also noted by Swift (1950). Measurements of the diameters of nuclei of the Feulgen and methyl green material showed that the irradiated nuclei had significantly larger diameters than the non-irradiated (Tables III

Table III

Data showing mean diameters of control and irradiated nuclei. Feulgen-stained section; Carothers fixation

Treatment	Hydrolysis time (min.)	Number of nuclei measured	Mean diameter (μ)
X-ray 4000 r	10	20	11.55
Control	10	20	10.29
X-ray 4000 r	20	40	11.85
Control	20	40	10.85
X-ray 4000 r	30	20	11.70
Control	30	20	10.22
X-ray 4000 r	40	20	11.50
Control	40	20	10.42

TABLE IV

Data showing mean diameters of control and irradiated nuclei. Methyl green smears; fixation in 4 per cent neutral formalin

Treatment	Number of nuclei measured	Mean diameter (μ)
X-ray 4000 r	30	10.13
Control	30	8.26
X-ray 10,000 r	20	11.15
Control	20	9.47

and IV). Nuclei that had received an x-ray dose of 12,500 r were not significantly larger than nuclei receiving only 4000 r. Therefore, the degree of swelling at 10 hours after treatment does not appear to be related to the dose of irradiation received. Living-culture experiments performed in this laboratory have shown that the neuroblasts of grasshopper embryos swell after receiving any dose greater than 100 r of gamma or x-radiation. This swelling is independent of the dose rate, as it was observed whether a dose of 100 r was given in 1 minute or in six days. A change in osmotic pressure accompanies the swelling of cells, since after irradiation the cells are hypertonic in a culture medium in which they were isotonic previous to irradiation.

TABLE V

Average amounts of DNA per nucleus in embryonic grasshopper tissue, as obtained by photometric determinations of Feulgen preparations *

	Arbitrary units of DNA			
	Carothers fixation (sectioned material)		Carnoy fixation (smears)	
	Control	Irradiated 4000 r	Control	Irradiated 12,500 r
	3.18	2.78	3.51	4.93
	2.80	3.23	2.99	4.99
	2.61	2.61	3.55	4.90
	6.22†	2.34	3.72	3.94
	3.15	2.19	2.86	2.48
	3.05	2.83	3.15	3.02
	3.16	2.89	3.27	3.44
	3.32	2.20	3.62	2.96
	2.91	3.32	3.92	3.28
	2.96	2.38	2.72	4.83
	3.00	2.87	3.08	4.52
	2.69	3.00	2.89	4.86
	2.73	3.23	3.59	2.83
	3.42	3.18	2.27	3.84
	3.23	2.22	3.42	3.69
	2.69			
Mean	2.99	2.75	3.24	3.90
Standard error	0.28	0.26	0.31	0.40

* Each value is an average of five readings on one nucleus.
† Not averaged with other values since this cell probably had 4N DNA content.

Feulgen determinations. Photometric measurements on tissue stained with Feulgen show, when corrections for nuclear volume are made, that approximately the same number of dye molecules exist in irradiated and non-irradiated nuclei. Statistically, there was no significant difference between the irradiated and control cells (Table V).

Methyl green determinations. Photometric data on methyl green stained material revealed a highly significant difference (1 per cent level) between the extinction values of the irradiated (4000 r and 10,000 r) and non-irradiated nuclei. The results are summarized in Table VI.

TABLE VI

Average amounts of DNA per nucleus in embryonic grasshopper tissue, as obtained by photometric determinations of methyl green preparations *

Arbitrary units of DNA

Control	Irradiated 4000 r	Control	Irradiated 10,000 r
6.34	3.11	4.45	2.66
5.92	2.44	2.29	1.90
4.23	2.13	5.66	1.89
4.86	1.79	6.74	1.45
4.57	1.85	3.48	0.88
6.02	2.55	5.83	1.46
5.36	2.02	5.22	1.78
5.86	2.43	3.46	1.83
5.55	3.01	2.99	1.48
6.00	3.02	6.42	1.96
4.23	1.87	3.25	1.83
4.86	1.58	3.48	2.02
4.43	2.31	2.92	2.42
5.57	2.46	4.54	2.23
4.69	2.98	6.83	2.41
4.36	2.72	6.47	1.87
4.15	2.83	5.25	2.36
6.55	1.89	5.83	1.84
Mean 5.20	2.39	4.73	1.90
Standard error 0.19	0.11	0.35	0.10

* Each value is an average of five readings on one nucleus.

With nuclei which had been made fairly homogeneous, it was difficult to distinguish between late telophase, interphase, and early prophase. Photometric measurements disclosed a few nuclei which had twice the DNA content of the other nuclei (Table V). The increased amount of DNA may be attributed to a polyploid nucleus resulting from a radiation-induced suppression of cytokinesis.

DISCUSSION

The present study has shown that an increase in nuclear size occurs after x-radiation. This has been observed in a wide variety of biological materials. Hol-

weck and Lacassagne (1930) found that yeast spores divided and then swelled after irradiation. Similar results were obtained by Robertson (1935) with protozoa; Lea, Haines and Coulson (1937) with bacteria; Mottram (1927) with mice tumors; Glücksmann (1941) with human tumors. Lea (1947) explained such cases, where the swelling did not appear for several hours or days after treatment, by stating that the effect was due to an inhibition of cellular division while growth continued at a normal rate. If our studies had been based entirely on stained material (fixed 10 hours after irradiation), Lea's explanation would have satisfied the data. However, our experiments with living tissue cultures have shown that swelling occurs in a matter of minutes after treatment. When the larger doses are given, the swelling is instantaneous. A normal growth rate can hardly account for such rapid swelling. Observations on irradiated tissue cultures lead us to believe that water is being absorbed rapidly by the cells, presumably as a result of an x-ray-induced osmotic pressure increase. Failla (1940) suggested that radiation might change the cellular constituents so that the osmotic pressure is increased without altering the permeability of the cellular membrane. The depolymerization or molecular rearrangements of DNA, indicated by the methyl green results, might then be a factor contributing to the increase of osmotic pressure and thus cause the nuclei to swell.

Probably the first study reporting that radiation could increase the nuclear volume without decreasing the amount of chromatin was made by Mottram in 1933 on work with bean root tips. After accurate and detailed visual observations he wrote (p. 218) that the increase in size of the nuclei after radiation "is due to a great increase in the nucleoplasm, the other constituents of the nucleus remaining normal in amount. The chromatin becomes thus more dispersed and has the appearance of being diminished in amount, but in reality it remains normal in quantity." Results suggesting that the nuclear volume may be changed considerably without a significant variation in the Feulgen-stainability of DNA, are in agreement with recent work by Schrader and Leuchtenberger (1950), and Leuchtenberger (1950). The former authors reported that the DNA per nucleus remained constant, despite an eight-fold increase of nuclear volume in the spermatocytes of *Arvelius albopunctatus*. Additional studies disclosed that an increase or decrease of nuclear volume is due mainly to a corresponding modification in the protein, RNA, and water content of the cell and not to an alteration of DNA. Leuchtenberger found in early pycnosis of tumor cells, a condition in which marked reduction of nuclear volume occurred, the Feulgen-stainability of DNA remaining unchanged in quantity. The conclusion seems justified that x-radiation may augment the nuclear volume without destroying the DNA desoxypentose.

The cytochemical studies using methyl green show that some physical property of DNA is affected. Probably depolymerization or some change of the molecular configuration has taken place, and the change is too slight to detect visually. Many investigators report that depolymerization of the DNA may occur after treatment with ionizing radiations. In 1939 Svedberg and Brohult suggested that, directly or indirectly, radiation splits the molecular constituents within the cell. The stickiness and loss of spiralization noted in chromosomes after irradiation may be produced by the depolymerization of nucleic acids (Darlington and La Cour, 1945). Sparrow and Rosenfeld (1946), and Taylor, Greenstein and Hollaender (1947) report x-ray effects on isolated DNA but conclude that the nucleic acid is not destroyed, since no chemical property is altered. Both investigations reveal a loss of

streaming birefringence and a great drop in viscosity of the DNA after x-ray treatment. Since both the magnitude of relative viscosity and the intensity of flow birefringence are indicative of the degree of molecular asymmetry, the above changes in these properties probably represent a depolymerization or rearrangement of the molecular particles.

Direct observations with a microscope of Feulgen-stained tissue show that nuclei which were given a dose of 4000 r or more are less intensively stained than non-irradiated nuclei. However, when nuclear measurements and light absorption readings are made, it is evident that there is not a significant decrease of DNA desoxypentose. The apparent visual loss of Feulgen stainability would seem to be the result of nuclear swelling, that is, a change in the distribution of the DNA in the nucleus. With microscopic observations the intensity and color of irradiated and control nuclei appear to be the same in tissue stained with methyl green, but photometric measurements disclose a highly significant loss in stainability after radiation.

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Summary

1. Cytological and cytochemical studies were made on embryonic nuclei of the grasshopper, *Chortophaga viridifasciata*, after x-ray doses of 4000, 10,000, and 12,500 r. The changes induced were photometrically measured by using (1) the Feulgen reaction to determine relative changes in the DNA desoxypentose, and (2) the methyl green stain to indicate the degree of polymerization of the nucleic acid.

2. X-radiation was found to cause swelling of the nuclei. When correction was made for this, the Feulgen-stained nuclei showed no significant loss of DNA after irradiation, but the nuclei stained with methyl green disclosed a highly significant loss of stainability. This is interpreted to indicate that x-rays do not destroy the DNA but induce depolymerization of the nucleic acid.

3. This study has shown that it is not safe to make quantitative estimates of DNA (stained by Feulgen or methyl green) from microscopic observations and photographs. The visually apparent loss of Feulgen-stainability after irradiation is probably not due to a decrease in the DNA desoxypentose but rather to the increased dispersion of the DNA in nuclei that have undergone x-ray-induced enlargement.

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EXISTENCE OF PHYSIOLOGICALLY-DIFFERENT RACES OF OYSTERS, CRASSOSTREA VIRGINICA

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Until recently it was generally accepted that the population of the American oyster, ***Crassostrea virginica*** Gmelin, which extends along the Atlantic Coast from Prince Edward Island, Canada to Florida, and inhabits the Gulf of Mexico, was composed of individuals of the same physiological race, or variety, showing the same physiological requirements, such as the lowest temperature at which they could begin spawning. This opinion was especially well crystalized by Nelson (1928a) in his discussion of the so-called "critical temperature for spawning of oysters," where he concluded that our oysters, as well as other lamellibranch mollusks, begin to breed at a definite temperature which is constant for the species. Somewhat later he (Nelson, 1928b) expresses this view even more precisely by stating (p. 154) that "The American oyster *Ostrea virginica* Gmelin spawns after the water temperature reaches 20° C., over all parts of its range, with no adjustment to the extremes of its distribution."

Nelson's opinion, with which most of the oyster biologists of that period agreed, prevailed uncontested until Hopkins (1931) and Loosanoff (1932) working at Galveston Bay, Texas, and James River, Va., respectively, showed that in those areas the temperature of 20.0° C. was reached far ahead of the ripening of gonads of oysters. Therefore, spawning of oysters there began at a considerably higher temperature, usually when it was near 25.0° C.

A further and, perhaps, decisive argument against the theory was raised when it was found that some of the oysters of Long Island Sound were spawning at temperatures ranging between 16.4 and 18.5° C. (Loosanoff, 1939). Thus, contrary to the established theory, it became clear that not all local groups of *C. virginica* of the range occupied by this species begin to spawn when the temperature of 20.0° C. is reached. Actually, different groups appeared to require different temperatures. This suggested that the oysters composing the different geographical groups may belong to physiologically-different races.

As far as we could establish, the first reference in the literature to the possibility of the existence of physiologically-different races within the population of the same species, *C. virginica*, was made by Loosanoff and Engle (1942). The suggestion was based upon the differences in the spawning behavior shown by the different groups of the oyster population of Long Island Sound. Several years later Loosanoff and Tommers (1948), while discussing the behavior of oysters in turbid waters, again mentioned that there appear to be physiologically-different races within the general population of *C. virginica*, some of which have developed a greater tolerance to turbidity than the oysters of Long Island Sound. Recently Stauber (1950) reviewed the rather limited material pertaining to the possibility of existence of physi-

ologically-different races of *C. virginica*, emphasizing the need for additional research in this field. The present article is our preliminary contribution to the solution of this interesting problem.

The first steps to determine experimentally whether oysters of different geographical areas show different physiological traits were taken in the fall of 1948. Groups of young oysters, which had metamorphosed that summer, were received from Warren, Mass., Cape May, N. J., Seaside of Va., James River, Va., Pamlico Sound, N. C. and Pensacola, Fla. They were suspended in large wire trays under identical conditions in Milford Harbor. A group of Long Island Sound oysters of the same age was placed under the same conditions to serve as the control.

The chief trait to be studied was the ability of the oysters of different geographical areas to discharge spawn in our waters, where the maximum water temperatures reached during the summers are usually considerably lower than those of more southern areas. Furthermore, in Long Island Sound the period of relatively high temperatures, 20.0° and above, is of rather short duration, seldom exceeding 8 or 9 weeks (Loosanoff, 1937), while in James River, Va., from where one of our southern samples came, such temperatures may persist from about May 10 to about October 20, a period of 160 days, or about 23 weeks (Loosanoff, 1932).

Observations on the North Carolina and Florida oysters were discontinued in the spring of 1949 because 87 per cent of the first, and 97 per cent of the second group died during the preceding winter. Death was tentatively ascribed to the prolonged low temperature under which the oysters remained, but the final conclusion regarding this matter will be formed only after additional observations on the survival of southern oysters in our waters are completed.

Detailed gross and histological examinations of the remaining oysters were made on September 27, 1950. Samples, each consisting of 20 specimens, were taken from each group. By that time, all the oysters involved in these studies had spent approximately two years in Milford Harbor and, thus, were considered as having fully recovered from any detrimental conditions that might have accompanied the shipping of the oysters from their place of origin to Milford. At the time of collection the temperature of the water was already down to 16.0° C.

The results showed clearly that the gonads of the oysters of the different groups were in decidedly different conditions (Table I). All the individuals comprising the two northern groups, representing the Massachusetts and Long Island Sound populations of oysters, were completely spawned out (Fig. 1). In most instances the gonads had already passed through the stage of resorption of pycnotic cells left after spawning, and the oysters were accumulating glycogen, a normal process closely correlated in local waters with seasonal gonadal changes (Loosanoff, 1942).

In contrast with the conditions found in northern oysters, the majority of those of the three southern groups still retained all or the largest part of their spawn (Table I and Fig. 2). A few, nevertheless, possessed completely or almost completely empty gonads, although it was not determined, with absolute certainty, whether those oysters were spawned out or their gonads were resorbed after failure to spawn.

In all three southern groups many oysters had creamy, soft gonads, the thickness of which was often as much as 3.0 mm. However, in the other oysters of the same groups the consistency of the gonadal layer was, regardless of its thickness, somewhat harder than usual indicating, perhaps, that cytolysis and resorption of the un-

discharged material was already in progress. This condition was even more pronounced in the oysters with partially full gonads, which, as a rule, were characterized by the presence of extremely large numbers of phagocytic cells. However, even in those oysters some of the spermatozoa and eggs were normal in appearance, the spermatozoa usually becoming active if placed in a drop of water.

The average thickness of the gonadal layer of the oysters of the three groups ranged from 1.2 to 2.3 mm., being the thickest in the James River, Va. lot which was the most southern group (Table I).

Attempts to induce spawning of the oysters of the three southern groups failed. None of the oysters released any spawn despite the fact that a combination of tem-

TABLE I

Conditions of gonads of oysters originated in different geographical areas, kept in Milford Harbor, Conn. for about 2 years. End of spawning season, 1950

Area of origin	Conditions of gonads			Gonad thickness, range in millimeters	Average gonad thickness in millimeters
	Unspawned	Partly empty	Empty		
September 27 (20 oysters per sample)					
Wareham, Mass.	0	0	20	0.0	0.0
Long Island Sound	0	0	20	0.0	0.0
Cape May, N. J.	7	12	1	0.0–3.0	1.2
Seaside, Va.	10	8	2	0.0–3.0	1.6
James River, Va.	15	3	2	0.0–3.5	2.3
October 25 (10 oysters per sample)					
Wareham, Mass.	0	0	10	0.0	0.0
Long Island Sound	0	0	10	0.0	0.0
Cape May, N. J.	2	3	5	0.0–3.0	0.6
Seaside, Va.	4	5	1	0.0–3.5	1.7
James River, Va.	4	6	0	1.0–3.0	2.1

perature and chemical stimuli was tried. The stimulated oysters moved their shells as in spawning but, nevertheless, released no eggs or sperm. It is possible that at that time the degenerative processes within the gonads had already progressed to such an extent that normal spawning became impossible.

The second examination was made on October 25, 1950 when the temperature of the water in Milford Harbor had decreased to 14.0° C. On that occasion, however, only 10 oysters per sample were taken. The examination again emphasized the differences between the northern and southern groups. The two northern groups were, of course, again found with discharged gonads and in the process of accumulation of glycogen. The southern groups, however, still contained a considerable quantity of spawn (Table I). In the Cape May, N. J. group, half the oysters contained all or some spawn. Active sperm could still be found but eggs, as a rule, although

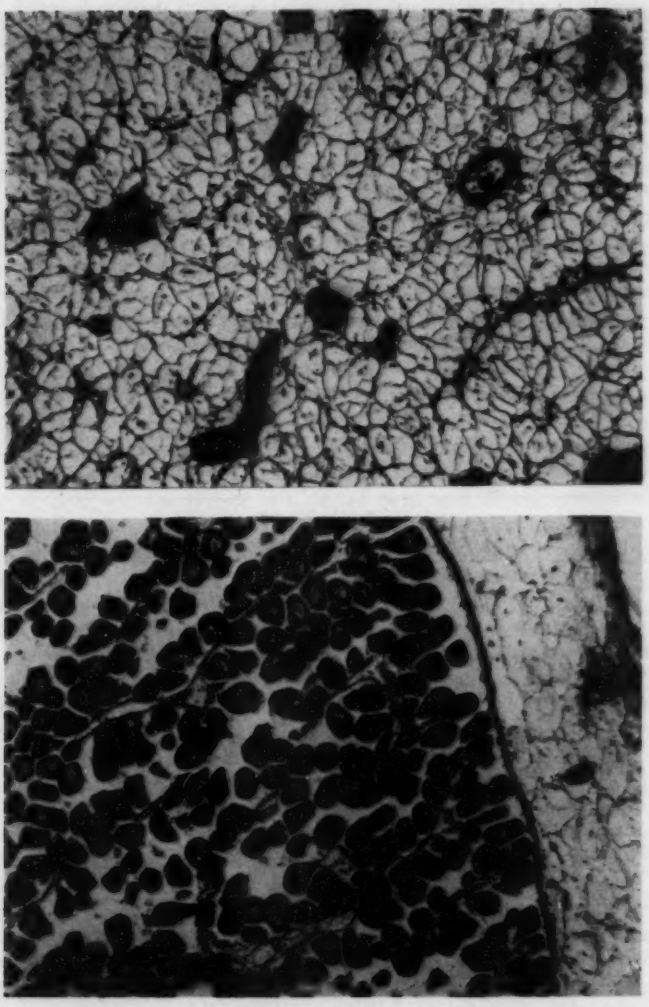

FIGURE 1. Section of gonad of Long Island Sound oyster preserved late in September, showing complete recovery from spawning. Small, undeveloped follicles, typical for post-spawning condition of oysters, are surrounded by masses of cells of the connective tissue. × 110.

FIGURE 2. Section of gonad of Virginia oyster preserved late in September, still containing fully developed undischarged eggs. × 110.

present in very large numbers, appeared to be abnormal. The color of the gonads changed from creamy white to grayish yellow, and instead of being soft and oozing, as was often noticed during the examination of September 27, the gonad material

was hard. In almost all instances resorption of gonad material was in progress, the number of leucocytes and phagocytic cells being extremely large.

The other half of the New Jersey oysters had their gonads completely or almost completely devoid of spawn. Whether these oysters discharged the spawn in a normal way or whether their gonads were resorbed after failure to spawn, remained undetermined.

Somewhat the same conditions were observed in the two Virginia groups. However, only one out of 20 oysters had completely empty gonads. The others had either entirely undischarged gonads, or gonads that still contained large quantities of spawn. It was quite clear that at least 8 oysters of the 20 had never spawned (Table I). The thickness of the gonadal layer of the unspawned oysters was 3.0 mm. or more.

Our controlled experiments in Milford Harbor were collaborated by observations made on the oyster beds of Long Island Sound. A schooner load of oysters native to the Seaside of Virginia, south of Chincoteague, was brought to Connecticut by F. Mansfield and Sons Oyster Company and planted on the cultivated beds of the Company in Long Island Sound, south of West Haven, at a depth of approximately 10 feet. Examination of the gonads of these oysters made on several occasions late in the fall showed that many oysters did not release their spawn, while the local oysters living on the same bed were completely spawned by the middle or the end of September.

In general, our experiments have shown that the oysters which originated in Massachusetts or Long Island Sound, *i.e.*, in the more northern latitudes of the range in which *C. virginica* is found, spawned in Milford Harbor, Conn. during the summer of 1950, discharging all the spawn. However, the majority of the oysters that originated in warmer regions, New Jersey or Virginia, either did not spawn at all or discharged only a portion of the accumulated spawn. This difference indicates that the breeding temperature requirements of the northern oysters are somewhat lower than those of the southern groups. It also suggests that some physiological requirements of oysters of different geographical districts may be decidedly different. Consequently, it may be logically assumed that these groups of oysters, although all belonging to the same genus and species, *Crassostrea virginica* Gmelin, may, nevertheless, be distinct physiological races.

If, as our observations seem to indicate, there are different physiological races among the population of *C. virginica* of our Atlantic Coast, which require different temperatures for spawning, then, in addition to the theoretical interest of these observations, we are immediately faced with a number of considerations which may arise in connection with transplantation of oysters from one geographical district to another, especially a far removed one. For example, it has been shown that the oysters of Long Island Sound may begin to spawn at a temperature as low as about 16.0° C. (Loosanoff, 1939). Were these oysters transplanted to southern areas, they, as pointed out by Stauber (1950), would, under the new temperature conditions, begin to spawn far ahead of the local population. It is true that since spawning of a single oyster may continue for several weeks, some of the northern oysters may, at the end of their spawning period, crossbreed with the southern ones, which, requiring a higher temperature, would begin spawning later in the season. Nevertheless, because of the absence in the water of gametes of southern oysters during

the largest part of the spawning of the northern races, most of the progeny of the northern oysters should remain to be of pure northern strain.

On the other hand, the majority of southern oysters transplanted to Long Island Sound, or other bodies of water in the north where the temperature seldom reaches and is practically never maintained for a prolonged period at 25.0° C., would not spawn. Such oysters, naturally, cannot be counted upon to contribute to the future increase of the local population.

Finally, the oysters with unspawned or partially resorbed gonads, of the type which were found in our samples of the southern groups on September 27 and October 25, 1950, would be virtually unsalable because of their poor quality. Obviously, the presence of southern oysters on the northern beds may not always be desirable because, while competing with northern oysters for space and food, they might neither participate in the increase of the local oyster population nor present a desirable food product.

In conclusion it should be mentioned that it is very fortunate for our studies that the summer of 1950 was comparatively cool, thus preventing the temperature of the water of Long Island Sound and particularly of Milford Harbor, from reaching an unusually high level, as happened during the record-breaking hot and long summer of 1949 when a temperature of 25.0° C. and higher was maintained for long periods. Under those conditions, of course, all oysters, including those of the three southern areas, would have spawned completely, leaving no evidence of the different temperature requirements of the different groups. In the past we found such to be the case with one group of oysters received from Eastern Bay, Maryland, which was kept in very shallow water in Milford Harbor near the shore where the temperature on some occasions was high enough to permit them to spawn normally.

We wish to express our thanks to our colleague, H. C. Davis, for his participation in the examination of gonads of the oysters, and to our friends in different parts of the country who were kind enough to ship us oysters for these studies.

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THE EFFECT OF PARROT FISH THYROID GLAND ON METAMORPHOSIS IN FROG TADPOLES

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The functions of the thyroid are not as well known in cold-blooded vertebrates as in birds and mammals. Thyroid experiments produce less striking results in lower vertebrates than in warm-blooded animals, with the exception of those concerned with metamorphic changes in amphibians. Perhaps because of this the number of experiments designed to determine what the gland does in fishes, amphibians and reptiles is relatively few. A better knowledge of what the thyroid does in these forms is desirable not only because such knowledge would enable us to interpret more completely the activities of these animals, but also because it might throw considerable light on any non-metabolic role that may be played by the thyroid in birds and mammals. As far as fishes are concerned, the diffuse nature of the thyroid gland in most bony fishes makes it difficult to study. Its scattered follicles can neither be removed to produce hypothyroid states nor can extracts of it be prepared that are free from contamination with other tissues. A large encapsulated gland has been described in the swordfish (Addison and Richter, 1932) but this material is difficult to obtain and no studies have been made with it. Several of the parrot fishes so common in subtropical and tropical waters also possess a large, encapsulated thyroid which can be removed free from surrounding tissues (Matthews, 1948), and these fish are fairly easy to obtain. A number of experiments have already been carried out on this material (Matthews and Smith, 1948; Smith and Matthews, 1948). The experiments presented here were designed to determine whether or not the parrot fish thyroid can accelerate metamorphosis in tadpoles, and, if so, whether this effect can be reduced or abolished by treating the fish with an antithyroid drug before removing the gland.

Material and Methods

Second year tadpoles of *Rana clamitans* collected in and near Williamstown were used as test animals. One experiment was run on animals collected in April, but in all other cases the tadpoles were collected in September and October. Individuals with hind limb buds of one to three mm. were selected and placed in small aquaria with ten animals and two and one-half to three liters of water in each.

Several of the external changes that occur as metamorphosis proceeds have been used to test thyroid preparations or other iodine-containing compounds. Shortening of the gut (Swingle, 1918), appearance of limb buds (Romeis, 1923), and decrease in volume (Gaddum, 1927) have all been used, as well as growth of hind limbs and decrease in total length. Some of these changes appear to be more accurate than others for quantitative tests, particularly the shortening of the gut. On the other hand reduction in total length and growth of the hind legs are easier to measure and can be made as often as desired on the same individual, and these external changes

were chosen as indicators of the rate at which metamorphosis was progressing in the present experiments. Measurements were made with calipers every seventh day, the animals lying on wet filter paper, and the progress of metamorphosis was then recorded by noting any increase in the hind limb/total length ratio.

Determination of the threshold level necessary to initiate metamorphosis was not made. With feeding methods it is difficult to estimate with any accuracy the amount eaten, and practically impossible to determine how much absorption of dissolved material occurs through skin and gills. For such quantitative determinations injection methods are preferable. These were abandoned in the present experiments after injection of thyroid preparations dissolved in basic solution had resulted in high mortality.

Thyroids of the rainbow parrot fish (*Pseudoscarus guacamaia*) were collected at the Bermuda Biological Station and dried either immediately in an oven at 50° C. or after preservation in acetone. Several glands from untreated animals were collected, and also one from a fish that had received ten daily injections of 20 cc. of a solution of propylthiouracil saturated at pH 8.0 before removal of the gland. After drying, the glands were pulverized, mixed with flour in the proportions of 300 mg. of thyroid powder to 1 gm. of flour, and enough water was added to form a thin paste which was then dried in thin sheets. This material was fed in excess by crumbling it into the tanks. Filamentous green algae or Anacharis were also added. At forty-eight hour intervals the aquaria were cleaned and fresh water and food were added. Control animals were given food prepared in the same way by mixing dried tail muscle or dried auricle with flour to form a paste which was allowed to dry as before.

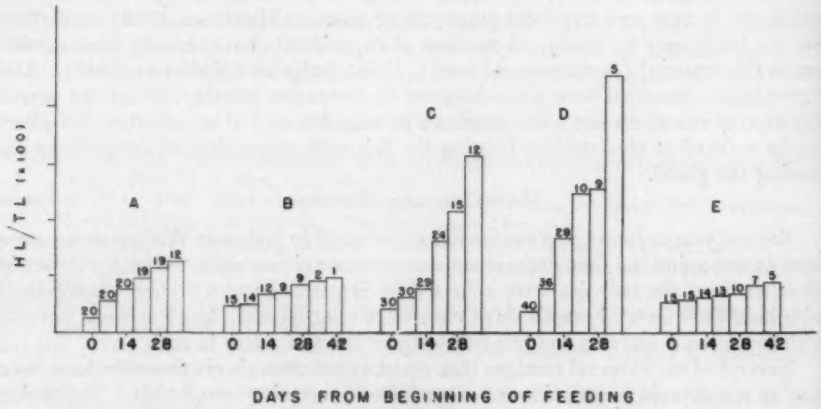

FIGURE 1. Rate of metamorphosis, as expressed by hind limb/total length ratios, of second year tadpoles of *R. clamitans* when fed preparations containing: A, Oven-dried fish muscle; B, Oven-dried parrot fish auricle; C, Oven-dried parrot fish thyroid; D, Acetone-dried parrot fish thyroid; and E, Oven-dried thyroid from a propylthiouracil-treated parrot fish. The figures at the top of the columns refer to the number of animals contributing to each average.

RESULTS

The effects of feeding these substances were studied on 130 tadpoles. Forty were fed oven-dried thyroid, 40 acetone-dried thyroid, and 15 were given thyroid from a propylthiouracil-treated animal. Of 35 control animals, 20 were fed tail muscle preparations; the other 15 were given food prepared from auricle tissue. The results on spring and fall animals showed some differences, but these could be interpreted as due to the small number of animals included in each experiment rather than to differences in threshold in the spring and fall tadpoles. At any rate the differences were not marked enough to justify separate presentation; consequently, the

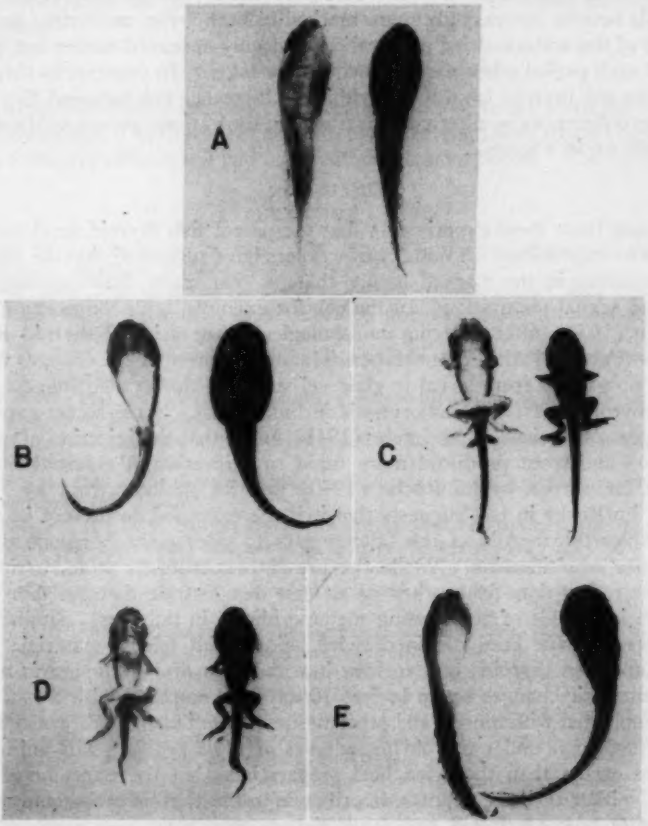

FIGURE 2. Second year tadpoles of *R. clamitans* after 28 days of feeding with preparations containing: A, Oven-dried fish muscle; B, Oven-dried parrot fish auricle; C, Oven-dried parrot fish thyroid; D, Acetone-dried parrot fish thyroid; E, Oven-dried thyroid from a propylthiouracil-treated parrot fish.

results of both spring and fall experiments are summarized and presented together in Figure 1. The height of each column represents the average ratio (hind limb/total length) of all animals in a given experiment on a particular day after feeding began. The number of animals contributing to each average is listed above each column. Since some animals died during the course of the experiments, the figures for the later parts of each run represent fewer cases.

Observable differences in the shape of the body of the tadpole occur late in the first week, the animal becoming more triangular in appearance. A change in the hind limb/total length ratio, as compared with that of the controls, also occurs at the end of the first week in the case of the acetone-dried preparations, but not until the third week with oven-dried thyroid. The differences between control and thyroid-fed animals became increasingly more marked in both series as feeding progressed. The effect of the acetone-dried preparations not only appeared earlier but was more marked at each period when measurements were taken. In contrast to these results the tadpoles fed thyroid from a propylthiouracil-treated fish behaved like the controls. The differences in external appearance in the various groups at the end of 28 days are shown in Figure 2.

Discussion

It is clear from these experiments that the parrot fish thyroid accelerates metamorphosis in amphibians. A number of workers have suggested that the fish thyroid may be involved in the marked bodily changes that many fishes undergo during growth and sexual maturation. In the eel, for example, both Sklower (1930) and von Hagen (1936), after studying morphological changes in the thyroid, concluded that it was concerned with, if not responsible for, the pronounced changes that occur as the eel progresses from larval to glass eel stage. Sklower also suggests that the flounder thyroid controls metamorphosis in this animal. Some recent experimental work supports this view. Landgrebe (1941) found that the injection of ox thyroid into salmon and trout produced more rapid smoltification, a result that has been confirmed for salmon by La Roche (1949) and for rainbow trout by Robertson (1949). La Roche in fact suggests that it is the extra iodine present in sea water which enables the thyroid of the salmon parr to release the hormone that is responsible for smoltification. While the present experiments were carried out on amphibians rather than fishes, they do at least demonstrate that the thyroid of the parrot fish is capable of accelerating metamorphosis in tadpoles. Similar but less marked results have been obtained twice before with teleost material. Sembrat (1927) implanted thyroids of Cyprinus into tadpoles of *R. temporaria* and found that metamorphic changes began in 5 to 10 days as compared with 8 to 23 days in controls implanted with muscle and other tissues. Gunthorp (1932) also tested this effect by feeding flounder thyroid to tadpoles of *Hyla regilla*. His animals metamorphosed earlier than those fed beef preparations, but the effect here, too, was slight. Neither of these reports describes how the thyroid was removed nor do they indicate how much gill and other tissues may have been removed with it. Contamination with inactive tissues may have diluted the concentration of active material in these cases and might explain the smaller effect produced by them than by the parrot fish thyroid.

Either iodine or potassium iodide will accelerate metamorphosis of amphibian larvae, but diiodotyrosine is much more effective than either, and thyroxine produces the most striking results of all iodine-containing compounds (Allen, 1938). No accurate figures are at present available for either the organically bound or total iodine content of fish thyroids. The correlation of their accelerating effect on metamorphosis with their iodine content must await chemical analyses of this material.

It has been clearly established for the higher vertebrates that propylthiouracil and other antithyroid drugs of this type prevent the synthesis of thyroxine, apparently by blocking the oxidation of iodide to iodine. Hence the thyroids of animals treated with these compounds contain only small amounts of iodine, most of which is inorganic. The results of the present experiments indicate that such drugs have a similar action in fishes, since the thyroids of propylthiouracil-treated fishes do not accelerate metamorphosis.

Summary

The parrot fish thyroid markedly accelerates metamorphosis when fed to tadpoles of *Rana clamitans*. The thyroid of a parrot fish which had been treated daily for several days with propylthiouracil had no effect on metamorphosis.

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A NEW COMMENSAL HYDROID OF THE MANTLE CAVITY OF AN OYSTER[1]

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During the course of studies on the biology of the "mangrove oyster," *Crassostrea rhizophorae* (Guilding), in Puerto Rico, a number of oysters were found with the mantle cavity infested by an undescribed commensal hydroid (Mattox, 1949). The unusual habitat of this hydroid and its correlated adaptive features are not entirely unique, for two similar hydroids have been found in Italy, both also living in the mantle cavity of edible pelecypods. The hydroid here considered is recognized as belonging to the same genus as those found in Italy and is designated as *Eugymnanthea ostrearum*.

The Genus Eugymnanthea

Palombi (1936) established the genus *Eugymnanthea* in naming a new species of hydroid found within the mantle cavity of the lamellibranch, *Tapes decussatus*, in Lago Fusaro, near Naples. This hydroid, *Eugymnanthea inquilina* Palombi is the generotype by monotypy. Cerruti (1941) described a similar hydroid from *Mytilus galloprovincialis* at Taranto. Unaware of Palombi's work he named this *Mytilhydra polimantii*, but upon learning of *E. inquilina* altered the name of his species to *Eugymnanthea polimantii*. Our species is similar to the other two and must be placed in the same genus.

The following are the features common to the three species of *Eugymnanthea* and constitute a definition of the genus. Athecate, solitary polyps, lacking hydrorhizae and gonothecae, possessing a basal disc, a single whorl of tentacles, and producing from near the base, either sporosacs, or medusae of the leptomedusan family Eucopiidae. The three known species are commensal within the mantle cavity of pelecypods.

Eugymnanthea ostrearum nov. sp.

Description of the hydroid (Fig. 1 A). The polyps are simple, solitary, tapering toward the base, having a maximum length of 3.8 mm. (the average of 20 specimens, taken at random, was 1.54 mm.), having a maximum diameter of the column of 0.35 mm. There are as many as 35 filiform tentacles (average 30) in a single whorl. There is a slight intertentacular membrane which, on the largest polyp, was 0.18 mm. in width. The hypostome is a slightly elevated cone which does not extend beyond the limits of the intertentacular membrane. A striking feature is the basal or pedal disc, a flattened, slightly concave expansion of the base of the column.

[1] Contribution from the Department of Biology, College of Agriculture and Mechanic Arts, Mayaguez, Puerto Rico (Mattox), and Contribution No. 474 from the Department of Zoology, Indiana University, Bloomington, Indiana (Crowell).

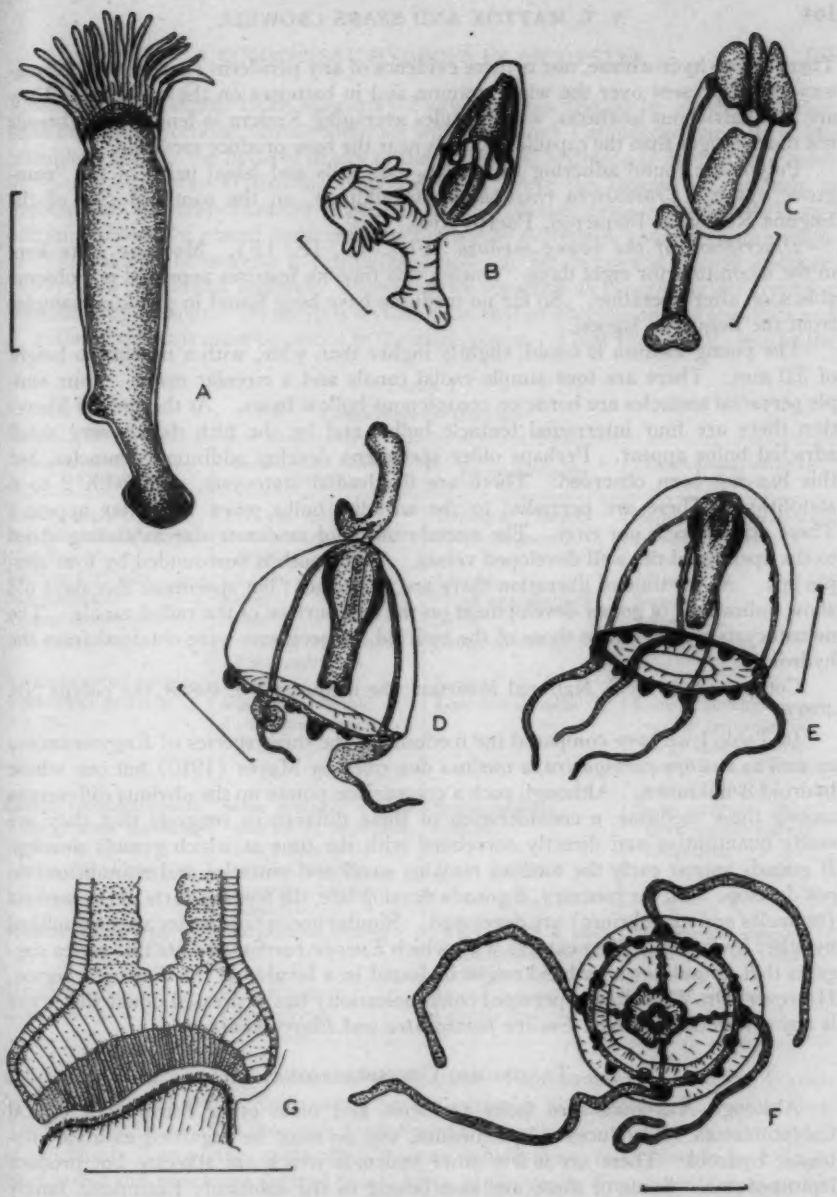

FIGURE 1. *Eugymnanthea ostrearum* nov. sp. A: Polyp. B: Polyp and late medusa bud; the oral region of the polyp shows an unusual eversion. C: Medusa bud just before it began to swim and at a stage when the polyp had become reduced. D: Medusa two hours after it became free swimming, with the polyp still attached. E: Medusa one day after liberation. F: Medusa in oral view five days after liberation. G: The basal disc on the ciliated epithelium of the oyster; the scale line is 0.1 mm. for G. For A to F the scale lines are 0.5 mm.

There are no hydrorhizae, nor is there evidence of any periderm (perisarc). Nematocysts are present over the whole column and in batteries on the tentacles. They are small atrichous isorhizas, with capsules averaging 5 micra in length, and threads not much longer than the capsules. Buds near the base produce medusae.

Polyps are found adhering to mantle, foot, gills and labial palps of the "mangrove oyster," *Crassostrea rhizophorae* (Guilding), on the southern side of the Laguna Rincon at Boqueron, Puerto Rico.

Description of the young medusa (Figs. 1D, 1E, 1F). Medusae were kept in the laboratory for eight days. During this time no features appeared not observable soon after liberation. So far no medusae have been found in plankton samples from the Boqueron lagoon.

The young medusa is ovoid, slightly higher than wide, with a maximum height of 3.0 mm. There are four simple radial canals and a circular canal. Four simple perradial tentacles are borne on conspicuous hollow bases. At the time of liberation there are four interradial tentacle bulbs, and by the fifth day 8 very small adradial bulbs appear. Perhaps older specimens develop additional tentacles, but this has not been observed. There are 8 adradial statocysts, each with 2 to 6 statoliths. (These are perradial to the adradial bulbs when the latter appear.) There are no ocelli nor cirri. The manubrium is of moderate size extending about to the opening of the well developed velum. The mouth is surrounded by four simple lips. At the time of liberation there are no gonads; but specimens five days old show indications of gonad development on the oral surface of the radial canals. The nematocysts are similar to those of the hydroid. Specimens were obtained from the hydroid.

Cotypes are in U. S. National Museum: the medusae No. 49808, the polyps No. 49809.

In Table I we have compared the medusae of the three species of *Eugymnanthea* as well as *Eucope parvigastra*, a medusa described by Mayer (1910) but one whose hydroid is unknown. Although such a comparison points up the obvious differences among these medusae, a consideration of these differences suggests that they are really quantitative and directly correlated with the time at which gonads develop. If gonads appear early the medusa remains small and tentacles and manubrium do not develop. On the contrary, if gonads develop late, the feeding parts of the medusa (tentacles and manubrium) are developed. Similar gonophore series are exemplified by other hydroids. The neatness with which *Eucope parvigastra* fits this series suggests that its unknown hydroid might be found in a bivalve of the Tortugas region. However, Dr. W. J. Rees (personal communication) has expressed doubt that there is any connection between *Eucope parvigastra* and *Eugymnanthea*.

Taxonomic Considerations

Although *Eugymnanthea* lacks periderm and most other features of typical Calyptoblastea, it produces a leptomedusa, and so must be regarded as a calyptoblastic hydroid. There are a few other hydroids which are athecate but produce leptomedusae. Some of these medusae belong to the subfamily Eutiminae, family Eucopiidae, and Stechow (1924) has proposed to place such hydroids in the subfamily Campanopsinae, of the family Heleciidae. This will not serve for *Eugymnanthea ostrearum* since its medusa belongs in the subfamily Obelinae of Eucopiidae.

Possibly (as Palombi, 1936, suggests) *Eugymnanthea* should be placed in a new subfamily of Haleciidae, having affinities with Stechow's Campanopsinae. It is impossible to include the hydroid in any of the families of hydroids as defined by Fraser in "North American Hydroids" (1937, 1944). Since the absence of periderm is probably an adaptive character related to the commensal mode of living, little significance should be placed upon it in attempting to determine natural relationships.

Fortunately the medusa is readily classified. It fits the genus *Eucope* (Mayer, 1910) in all anatomical features. However, we regard it as generically different for the following reasons. Its polyp is so similar to that of *Eugymnanthea inquilina* and *E. polimantii* that it must be placed in the same genus. Their medusae do not fit the

TABLE I

Comparison of Medusae

Eugymnanthea inquilina Palombi	*Eugymnanthea polimantii* Cerruti	*Eucope parvigastra*	*Eugymnanthea ostrearum* nov. sp.
Naples, Italy	Taranto, Italy	Tortugas, Florida	Boqueron, Puerto Rico
Polyp in mantle cavity of *Tapes*	In mantle cavity of *Mytilus*	Polyp unknown	In mantle cavity of *Crassostrea*
Medusa 0.55 mm.	1.2 mm.	1.0 mm.	3.0 mm.
Spheroconical	Same	"Half-egg-shaped"	Same
8 adradial otocysts	Same	Same	Same
Not reported	3 otoliths, sometimes 1 or 2, rarely 4	Single spherical	2 to 6 otoliths
4 simple radial canals	Same	Same	Same
4 perradial tentacle bulbs	4 perradial bulbs 4 small interradial bulbs	4 perradial bulbs	4 well developed perradial tentacles on large bases, 4 interradial bulbs, 8 small adradial bulbs
Gonads on radial canals. Large oval, shedding gametes before or soon after liberation	Same position. Same size as *E. inquilina*, hence relatively smaller, shedding gametes after liberation	Same position. Small	Same position. Not developed at time of liberation
Manubrium lacking	Manubrium lacking	"Very small, a mere tube cruciform in cross section"	Manubrium simple, extending to velar opening
Mouth lacking	Mouth lacking	Mouth present	Mouth present
Lips lacking	Lips lacking	4 simple lips	4 simple lips

definition of *Eucope* but are similar to *Agastra*. Furthermore none of the known hydroid stages of either *Agastra* or *Eucope* resemble the hydroid *Eugymnanthea*. Therefore we believe that the name *Eugymnanthea* should be applied to those medusae of the family Eucopiidae, subfamily Obelinae, which show resemblences to *Eucope* or *Agastra* and which are derived from polyps of the genus *Eugymnanthea* as previously defined.

REPRODUCTION OF THE HYDROID GENERATION

The method by which polyps produce other polyps has not been studied carefully in any of the species of *Eugymnanthea*. During the spring and early summer

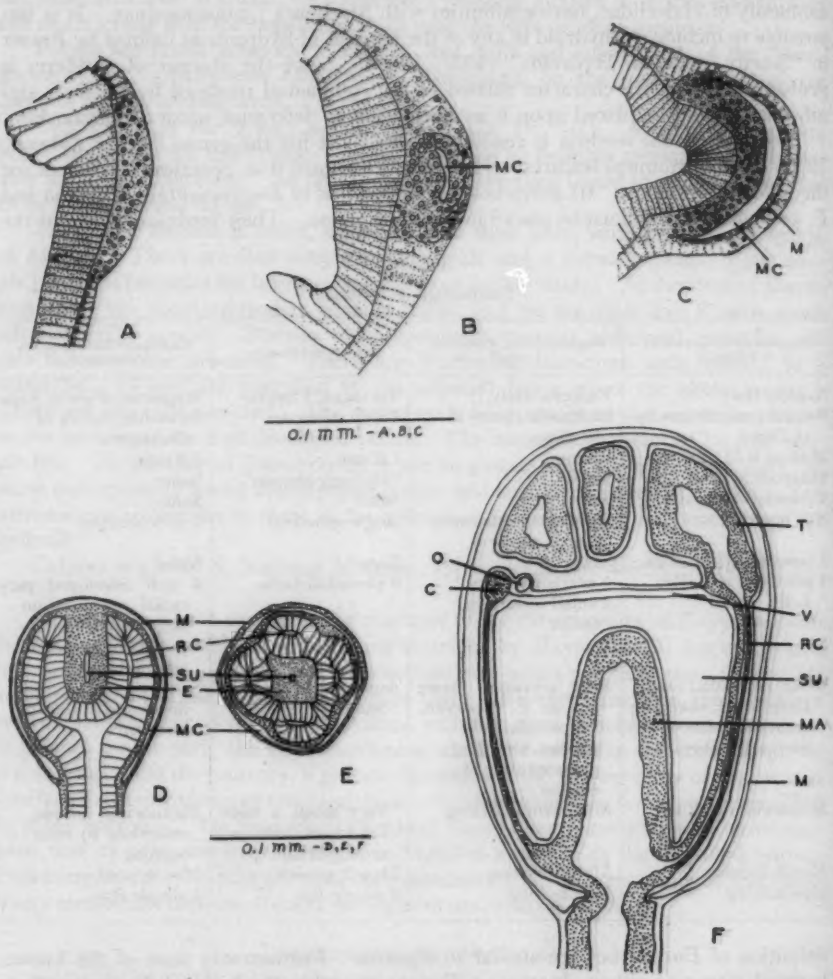

FIGURE 2. Development of the medusa. A: Early epidermal placode. B: Establishment of mantle cavity within the epidermal placode. C: Evagination of the bud and extension of the mantle. D, E: Diagrammatic radial and transverse sections of a stage at which the prospective sub-umbrella cavity is forming within the well established entocodon; epidermis stippled. F: Medusa just prior to liberation; on the left adradial section to show endodermal lamella and statocyst, on the right perradial to show radial canal and tentacle base; endodermis stippled. c, circular canal; e, entocodon; m, mantle; ma, manubrium; mc, mantle cavity; o, otocyst; rc, radial canal; su, prospective or actual sub-umbrella cavity; v, velum.

Cerruti (1941) found groups of polyps, usually two or three, once as many as 7, attached to one another and arising from one basal disc. He says that these arise by budding and that later the polyps separate from one another. Palombi (1936) does not mention this type of reproduction but does show figures of two and of three polyps arising from a common basal disc. In some of our specimens a small polyp is joined to a larger one near the base of the latter. In others two polyps of nearly equal size share a common basal disc. Lacking direct continuous observations we cannot be positive, but all indications suggest the following method of reproduction. The second polyp arises as a bud near the base of the first, just as in related campanularians. The bud becomes a polyp which grows. Finally the two polyps split the basal portion of the column and the basal disc so that when separation occurs each obtains part of the basal disc. Such a combination of budding followed by fission does not occur in any other hydroid, as far as we know, but it is appropriate for a species in which the basal disc is so important.

Development of eggs has not been observed for any species of *Eugymnanthea.* However, Cerruti (1941) observed, in October, on the mantles of some of the mussels some little, somewhat spherical bodies and some very small polyps. He interprets the small bodies as due to the attachment of larvae which would soon transform into polyps.

Development of the Medusa

Medusa buds arise singly near the basal end of the column, a polyp bearing only one medusa at a time. The changes of form and structure during development are similar to those described for other medusae having a comparable structure at the time of liberation. Since the other species of *Eugymnanthea,* unlike *E. ostrearum,* have gonads but no manubrium nor tentacles when liberated, it follows that our species is more like *Clytia,* for example, than it is like the other two species of *Eugymnanthea,* in respect to its developmental stages. (For *Clytia* see Goette, 1907.)

In the earliest stages of bud formation both epidermis and gastrodermis become modified. The epidermis becomes thickened as a placode (Fig. 2A). Its cells become less vacuolated and several layers deep. The gastrodermal cells beneath this placode have about the same height as those which lie between it and the basal disc, but are narrower. They have lost the vacuoles of typical gastrodermal cells but retain the conspicuous granules. By the time the placode has thickened so that it is about five cells thick, its cells have lost the vacuoles, and the underlying gastrodermal cells no longer show the large granules (Fig. 2B). The small size of the cells, their loss of vacuoles and granules, the increase in size of the placode and its evagination as a young bud all reflect high mitotic activity. This has been clearly shown for other developing medusa buds by Berrill (1949a, 1949b).

The mantle and mantle cavity are established very early through the rearrangement of epidermal cells of the placode. The mantle (sheath, ectotheca) is an epidermal covering of the developing medusa characteristic of most calyptoblasts. It is described as arising by delamination of irregular thickenings of the epidermis (Goette, 1907). In *Eugymnanthea* this delamination has more orderliness and precision than has been reported in other cases. Figure 2B shows an early mantle cavity. The cells which line the cavity appear to be oriented with reference to it,

and its extension the result of their rapid multiplication. The mantle itself is two cell layers thick. The outer cells soon become vacuolated and continuous with the epidermal cells beyond the placode; while the inner cells resemble those of the rest of the placode with which they are continuous around the circumference of the mantle cavity (Fig. 2C). The mantle becomes thinner as the medusa develops and is ruptured at the time of liberation.

The epidermal cells of the placode soon alter their arrangement to produce the entocodon within which the prospective sub-umbrella cavity is formed. Figures 2D and 2E illustrate this stage. The entocodon is quadrilateral in cross section. It is flanked on its slightly concave sides by the endodermal sacs destined to form the radial canals, and is bordered at its corners by a single layer of endodermal cells which are continuous with the endodermal sacs. Subsequently the sub-umbrella space expands; the manubrium arises and extends into it. By these processes and the establishment of tentacle bases, velum and otocysts, the stage is reached, just prior to liberation of the medusa, illustrated by Figure 2F. The four tentacle bases are much larger than in the other species of *Eugymnanthea* and most other calyptoblasts. They have about the same proportions as those of *Clytia* (Goette, 1907).

During the late stages of the development of the medusa there is a striking reduction in the size of the polyp. It loses its tentacles by reduction and resorption, and diminishes in size to less than one fourth of the original size. When the medusa ruptures its mantle, begins to pulsate, and becomes free swimming it carries the small remnant of the polyp with it. This remnant drops from the medusa soon after the medusa begins its free life. The reduction of the polyp is illustrated in Figures 1C, 1D, and 1E. Palombi (1936) found a similar reduction of the polyp during the formation of the medusoids of *E. inquilina*, but Cerruti (1941) found no such reduction in *E. polimantii*. An analagous reduction occurs in *Obelia* and in *Campanularia* (Berrill, 1949b; Crowell and Rusk, 1950), where blastostyle production often causes regression of the nearby hydranths. A similar regression of the polyp of *Craspedacusta* accompanies the production of a medusa (Payne, 1924).

In most colonial calyptoblastic hydroids the production of a gonangium requires the cooperative support of many feeding hydranths. A single small polyp, like that of *Eugymnanthea*, is probably unable to furnish sufficient substance for the construction of a gonangium. As an alternative it produces a single medusa with a morphogenesis like that of one of the medusae arising on a blastostyle.

The Commensal Relationship

Oysters containing *Eugymnanthea* have been found at all seasons in the Boqueron lagoon. In 11 random samples, totalling 138 oysters, and extending from January to December of 1950, 12 per cent contained polyps. In heavily infested oysters the polyps are attached chiefly to the inner surface of the dorsal region of the mantle, but are also found on the foot, gills and labial palps. Some are scattered and isolated, others are found in large groups. Some oysters contain only a few polyps, others many. In one oyster 4.5 cm. long, 210 polyps were counted.

The most striking feature of *Eugymnanthea* is the conspicuous basal disc by which the polyps are enabled to remain attached to the host tissue. In detached specimens the disk appears circular and concave. In prepared sections, where it is attached,

it shows both a tendency to be concave and at the same time to modify its shape to conform with folds of the host epithelium. The epidermal cells of the disc are high, narrow, heavily stained (hematoxylin), and non-vacuolated (Fig. 1G). The gastrodermal cells of the disc are large and lack the conspicuous granules of unmodified gastrodermis. Although the disc resembles the pedal disc of hydra superficially, it seems clear that the ability to adhere is not due to secretion of a mucous material which could hardly be effective on the slimy epithelium, but rather to suction achieved through the muscular activity of one or both cell layers. In living oysters the polyps sometimes become detached, then re-attach at other sites.

Specimens are attached both on naked and on ciliated epidermis of the host. The cilia do not appear to be damaged. There is no evidence of penetration into host tissue nor alteration of it. Cerruti (1941), in *E. polimantii*, reports loss of cilia of the mantle where the discs attach, and the presence of granules in the cytoplasm of the epithelial cells of the host mussel. The disparity between our observations and his may be due to a real difference between the hydroid-host relationship in the two pairs of species, or it may be that such damage may sometimes occur, sometimes not, in both species.

The mantle cavity of a pelecypod is obviously an excellent dwelling place for a small hydroid. It provides protection and an abundant current of water. The only disadvantage would seem to be the difficulty in remaining there. Most hydroids could not do so; *Eugymnanthea* has the necessary adaptation, the basal disc. Periderm (perisarc) is unnecessary in such a small and well protected polyp. Stolons (hydrorhizae) would be useless. The modifications in the methods of producing additional polyps and in producing medusae, discussed earlier, are also appropriate to the peculiar habitat of this hydroid.

We wish to thank Dr. W. J. Rees of the British Museum for helpful suggestions, particularly in reference to the taxonomy.

Summary

1. *Eugymnanthea ostrearum* nov. sp. is a small, solitary hydroid living, attached by a basal disc, within the mantle cavity of the oyster *Crassostrea rhizophorae* (Guilding), in Puerto Rico. Two similar species have been described from Italy.

2. Its taxonomic position as a hydroid is somewhat uncertain since it lacks nearly all of the characters used in hydroid classification. It produces a leptomedusa similar to *Eucope*, and as a medusa belongs to the family Eucopiidae, subfamily Obelinae.

3. It shows the following features which may be regarded as adaptations appropriate to its commensal existence within the mantle cavity of the oyster: basal disc, absence of perisarc and stolons. Since the polyps are solitary there can be no gonangia.

4. Polyps are produced by budding followed by a fission of the basal disc. The development of the eggs has not been studied.

5. The development of the medusa is not unusual except in the way in which the mantle is established. It arises in a more sharply defined manner than that described in other forms by previous observers.

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THE SOLUBILITIES OF SOME VERTEBRATE FIBRINOGENS IN PLASMA–ETHANOL MIXTURES[1]

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Fibrinogen, the plasma protein which forms fibrin under the action of thrombin, is the least soluble major component of plasma. It is a euglobulin precipitating when the ionic strength is reduced much below the physiological level and is "salted out" of mammalian plasmas at such relatively low ionic strengths as half saturated (3.2 *M*) sodium chloride (Hammarsten, 1879; Florkin, 1930) and 10.6 per cent (0.75 *M*) sodium sulfate (Howe, 1923). It is also precipitated in dilute solutions of ethanol at low temperatures (Edsall, Ferry and Armstrong, 1944; Morrison, Edsall and Miller, 1948) and we have used this behavior as a criterion of solubility.

Materials and Methods

Fibrinogens from representatives of four vertebrate classes were studied. Blood was usually obtained by exposure of the heart and direct heart puncture, although some animals were bled by heart puncture through the chest and others from the canulated carotid artery. Siliconed needles and syringes containing a tenth volume of 10 per cent sodium citrate as an anticoagulant were used. Blood was chilled on ice usually within 10 minutes and stored in siliconed or plastic containers until centrifugation, which was carried out in two stages with the second at 5000 r.p.m. to insure removal of fine particulate material which would otherwise be carried down in the clot. This procedure usually produced a very clear straw or yellow plasma although in certain individual frogs and turtles a substantial amount of hemoglobin was present in the plasma even with these precautions. This hemolysis was not correlated with differences in technique, so it may represent differences in the physiological condition of the animals about whose background little was known.

Since as little as one cc. of blood was the most that could be obtained from certain of the smaller animals, it was necessary to adapt procedures to small volumes. One cc. of plasma was diluted with an equal volume at an appropriate concentration of ethanol (by volume) and sodium chloride to maintain the ionic strength constant at 0.15 and the mixture was equilibrated for two hours at 0° C. The precipitate and supernatant phases were then separated by refrigerated centrifugation in the same tube and decantation. The precipitate was dissolved in 2½ cc. of pH 6.4 phosphate buffer, ionic strength of 0.15, and two 1 cc. samples of this solution together with a 1 cc. sample from the supernatant were analysed. The fibrinogen was assayed gravimetrically after clotting with thrombin, syneresis and washing, drying and weighing

[1] This is paper 6 of a series on "The formation of fibrin and the coagulation of blood" from the University of Wisconsin, supported in part by research grants from the National Institutes of Health, U. S. Public Health Service. This work was also supported by research grants from the Wisconsin Alumni Research Foundation.

(Morrison, 1947). Since the final clot weights usually ranged from 0.1 to 1 mg., a torsion balance sensitive to 0.005 mg. was employed. With these small amounts results usually agreed within 5 per cent. This represents a substantially greater error than had been obtained with 20 mg. clots (Morrison, 1947) of human fibrinogen. For mammalian fibrinogens a final concentration of 0.1 to 0.2 unit (Ferry and Morrison, 1947) per cc. of bovine thrombin [2] was employed. Bovine thrombin was also employed to clot fibrinogens of lower forms but since the heterologous reaction between fibrinogens and thrombins from different classes is much less effective (unpublished observations) larger amounts were necessary. A concentration sufficient to yield a 5 to 10 minute clotting time was usually employed and clotting was continued for two to four hours. Considerable fibrinolytic activity was sometimes encountered in plasmas from the lower forms and it was necessary to discard such series.

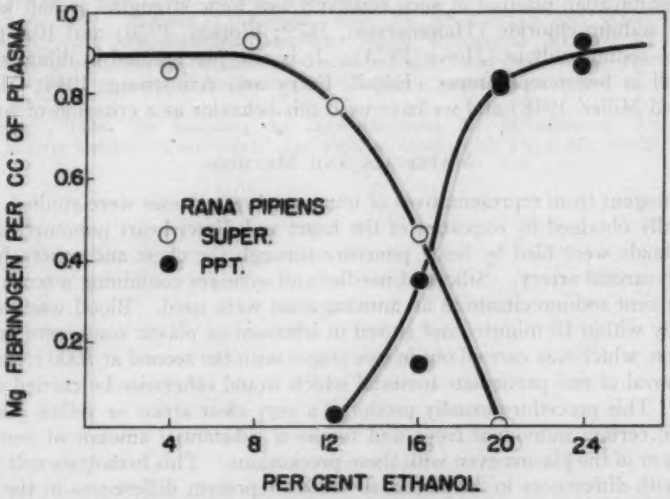

FIGURE 1. A representative series on 1 cc. samples of plasma showing the distribution of fibrinogen in the precipitate and supernatant phases with increasing amounts of ethanol. Open circles, supernatant phase; closed circles, precipitate phase.

Figure 1, showing the yield of fibrinogen in the supernatant and precipitate as a function of ethanol concentration, illustrates a typical experimental series on frog plasma using a total of 7 cc. of plasma. The sum of the supernatant and precipitate values usually was satisfactorily constant although at low fibrinogen levels low yields will be obtained (Morrison, 1947). For example in Figure 1 at 12 per cent ethanol this sum is only 90 per cent of the expected value. At high ethanol concentrations the yields tended to rise above the initial value presumably because of precipitation and/or denaturation of other plasma components with subsequent occlusion in the clot. In such cases the synerized clots were distinctly yellower.

[2] This material was supplied through the kindness of Dr. James Lesh of Armour and Company.

Results and Discussion

Precipitation curves for three species of mammals, the rabbit, dog and cow, are shown in Figure 2. The points are sufficiently homogeneous for a single curve to represent the three species. Actually, successive series on individuals yielded as much variation as is shown between species in this figure. Human fibrinogen, which is 65 per cent precipitated at 8 per cent ethanol, fits quite well with these series.

Results on two turtles, the snapping turtle, *Chelydra serpentia* and the terrapin, *Pseudemys*, are shown in Figure 3. Reasonable correspondence is seen between individuals but there is a distinct difference between the two genera.

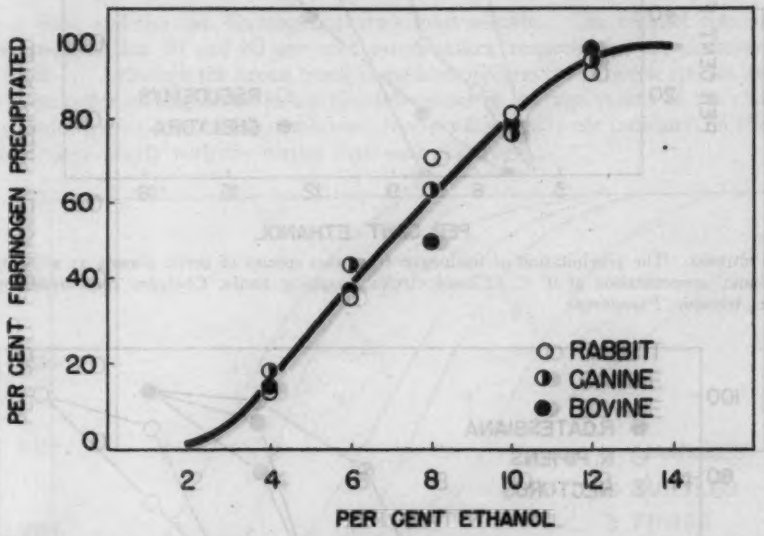

Figure 2. The precipitation of mammalian fibrinogens from their plasmas (per cent of the plasma level) as a function of ethanol concentration at 0° C. Open circles, rabbit; half circles, canine; closed circles, bovine. Each of the values for rabbit and dog represents an average of two series on different individuals.

Results on three amphibia, the bullfrog, *Rana catesbiana*, the green frog, *Rana pipiens*, and the mudpuppy, *Necturus*, are shown in Figure 4. Again, individuals of the same species fall together but distinct differences are seen between groups. The curve for the bullfrog suggests that two components with different properties were being precipitated, since 5 to 15 per cent of the material came down between 4 and 8 per cent ethanol while the bulk of the fibrinogen required considerably higher amounts of alcohol for precipitation.

Average curves for the species studied are assembled in Figure 5. The shapes of these curves are very similar although minor differences may be noted. It is immediately seen that the precipitation curves for the several classes stand in the same regular series which relates them phylogenetically, the mammalian fibrinogen

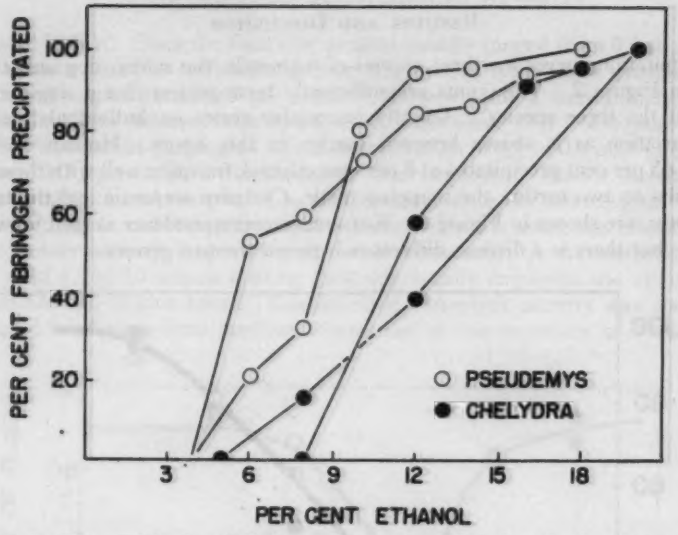

FIGURE 3. The precipitation of fibrinogen from two species of turtle plasma as a function of ethanol concentration at 0° C. Closed circles, snapping turtle, *Chelydra serpentina;* open circles, terrapin, *Pseudemys.*

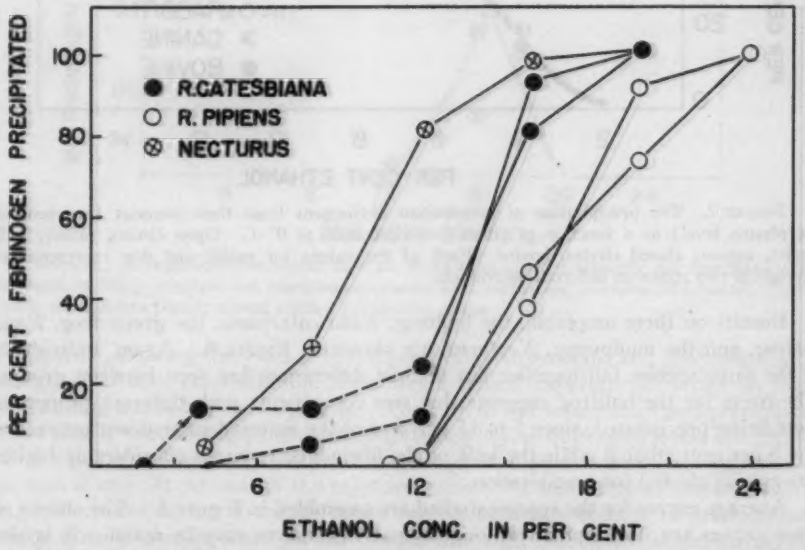

FIGURE 4. Precipitation of several amphibian fibrinogens from plasma as a function of ethanol concentration at 0° C. Open circles, grass frog, *Rana pipiens;* closed circles, bullfrog, *Rana catesbiana;* cross circles, mudpuppy, *Necturus.*

TABLE I

Average ethanol concentrations for precipitation of various fibrinogens from plasma

Group	Species	Series	% Ethanol for	
			50% ppt.	80% ppt.
Mammals	3	5	7	9
Turtles	2	4	11	13
Frogs	2	4	15	18
Carp	1	1	19	24

being least, and the fish fibrinogen (carp) most soluble. The ethanol concentrations required for 50 and 80 per cent precipitation, respectively, are summarized in Table I. Although the broad trend is evident, differences between species are of the same order of magnitude as are the differences in average values of the classes. And, indeed, the curve for the amphibian, *Necturus*, which is not indicated on Figure 5, falls more nearly with the turtles than with the frogs.

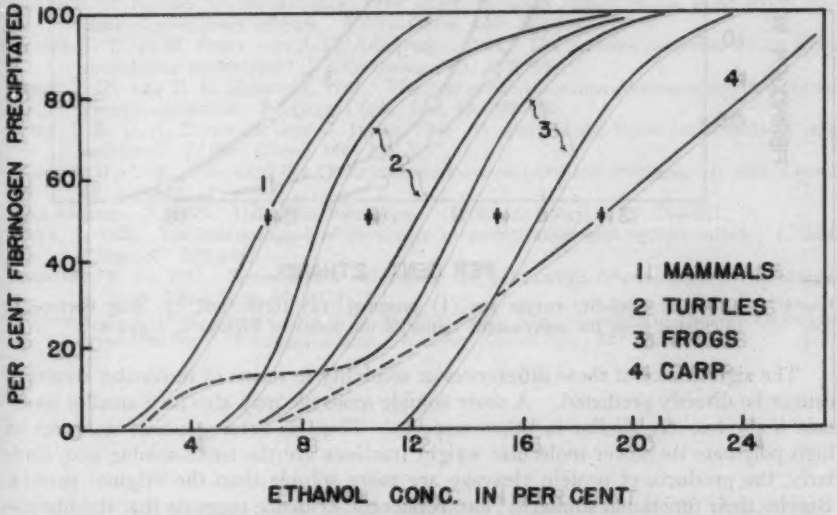

FIGURE 5. Average curves for the precipitation of fibrinogen as a function of ethanol concentration for the species shown in Figures 2, 3, and 4, together with a single curve for the carp. Symbols show the average values for 50 per cent precipitation for the four classes.

Although these precipitation curves reflect changes in solubility, they are not the same as solubility measurements since they will be influenced by the amount of the fibrinogen originally present in the plasma. However, since the curves are quite steep, variation in initial fibrinogen level will have a relatively small effect on the ethanol concentration required for 50 per cent precipitation. In Figure 6 the average results of measurements on the three classes have been presented in terms

of actual solubility of fibrinogen in the supernatant phase at successive ethanol concentrations. The trend toward increasing solubility in the lower forms is again shown.

It is worth noting that this trend in fibrinogen solubility appeared to be followed by the other components since the mass of the fibrinogen-containing precipitate in the lower forms was not substantially larger than in the higher forms despite the considerably higher ethanol concentration. Plasma samples from two primitive fish, the garpike and the bowfin, which were unsatisfactory for fibrinogen assay, showed almost no precipitation even at 25 per cent ethanol.

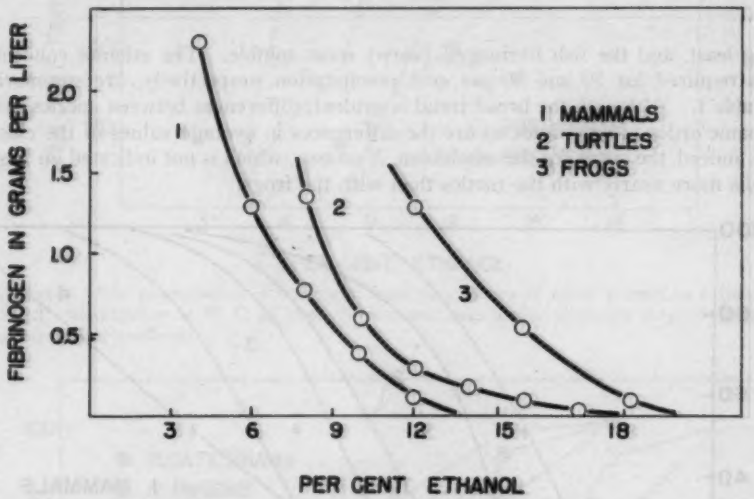

FIGURE 6. Average solubility curves for (1) mammal, (2) turtle, and (3) frog fibrinogens calculated from the supernatant values of the series in Figures 2, 3 and 4.

The significance of these differences in solubility in terms of molecular structure cannot be directly predicted. A more soluble molecule may also be a smaller molecule if the two are similar in other respects. Thus, in heterogeneous mixtures of high polymers the lower molecular weight fractions are the more soluble and, similarly, the products of protein cleavage are more soluble than the original protein. Besides their functional similarity, electrophoretic evidence suggests that the fibrinogens may be chemically similar. Deutsch and Goodloe (1945) reported a mobility of 2.6×10^{-5} cm.^{2} v^{-1} sec.$^{-1}$ for carp fibrinogen as compared to an average value of 2.43 (range: 2.1 to 3.2) for 12 species of mammals. Deutsch and McShan (1949) found no general shift toward components of higher mobility in studies on serum proteins from snakes, frogs and fish. However, their carp serum showed a shift toward components of higher mobility, perhaps correlated with the high albumin to globulin ratio reported for this species by Field, Elvehjem and Juday (1943). Such behavior, by reflecting a higher net charge on the molecule, might also account for the observed differences in solubility. Another piece of evidence which may suggest

smaller molecules in the lower forms is the fact that the clots formed from these fibrinogens are invariably of the "fine" variety (Ferry and Morrison, 1947) showing little turbidity and synerizing with difficulty. These properties are considered to indicate small units of structure and small interstices such as might be expected from an aggregation of smaller molecules. Final conclusions must, however, await more careful characterization of these fibrinogen molecules by physical and other means.

Summary

The precipitation and solubility of fibrinogens from plasmas of four vertebrate classes by ethanol at 0° C. and 0.15 ionic strength was investigated. A regular series showing increasing solubility was observed as one went from mammals down through the lower classes.

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NITROGEN TENSION IN THE SWIMBLADDER OF MARINE FISHES IN RELATION TO THE DEPTH

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It has long been known that fishes with gas bladders occur down to very considerable depths. At 20 atmospheres depths they are still very common. Ling has been caught at 300 fathoms (60 atm.), cusk at 500 fathoms (100 atm.) (Bigelow and Welsh, 1924). Richard (1895) gives gas analysis data on fishes trawled for and possibly caught as deep as 1674 meters (170 atm.). Schloesing and Richard (1896) give analyses from fishes trawled at 900 and 1385 meters depths.

The gas pressure in the sea is only about one atmosphere at all depths. Since the free gases in the swimbladder are compressed according to the hydrostatic pressure, it is clear that we are dealing with gradients in the gas pressure between the outside water and the inside of the swimbladder of 20 to 50 to 100 and possibly more atmospheres. Obviously, therefore, at least one of the gases in the swimbladder must be secreted against very high pressures. Any general theory wishing to explain the gas secretion into the swimbladder must be able to account for this fact. We shall not enter into any discussion of the different theories advanced to explain the oxygen secretion, as they have been excellently reviewed in a paper by Koch (1934). It suffices here to point out that even the most generally accepted theory (Jacobs, 1930–34) requires an extrapolation of at least ten times beyond present knowledge in order to account for the known pressure performance of the gas gland.

It is well known that fishes at the surface generally have a gas composition in their swimbladder near that of atmospheric air (Jacobs, 1930; Hall, 1924; Black, 1942). It has also been known for a long time that, in general, the oxygen percentage increases as the depth gets greater. Biot (1807, quoted by Tower, 1901) reports 87 per cent oxygen in *Trygla lyra* from 500 fathoms, and high oxygen values in several other species from lesser depths. Tower (1901) verified these findings in other species. Richard (1895) and Schloesing and Richard (1896) found 75–85 per cent oxygen in the swimbladder of several deep sea fishes mentioned above. In other cases, however, a very low oxygen concentration was found in fishes caught at 7–8 atmospheres depths (Hüfner, 1892). It seems that no systematic study has so far been made of the relation of depth to the gas composition of the swimbladder. Such knowledge is necessary for the characterization of the performance

of the gas gland. We have hence undertaken a study of the relation of depth to the partial pressure distribution of the gases of swimbladders from a variety of saltwater fishes.

Material

The present investigation was conducted from U. S. Bureau of Fisheries, Woods Hole, Mass., and from the Lerner Marine Laboratory, Bimini, Bahamas. The northern material was obtained on board MS Albatross III, Cruise No. 38, August 22–31, 1950, off the New England coast. The fishes were caught by bottom trawl which would drag the bottom for 30 minutes. It would take from 15–30 minutes from the time the trawl left the bottom until the gas sample was secured. Since the trawl was open on its way down and up, some fishes might have been caught in midwaters. The bottom depth was accurately known from a continuously recording fathometer. The shallow water fishes were caught by dragnet on shallow beach flats or by means of hook and line around Woods Hole.

Because of the comparatively long time which elapsed from the time the fishes were caught in the trawl to the taking of the sample, and because of possible asphyxial conditions in a fish-crammed trawl bag, we extended our work to include also a series of hand-lined fishes. This was undertaken at the Lerner Marine Laboratory at Bimini Island in the Bahamas during December 22 to January 3, 1951. Here the fishes caught down to 220 meters were immediately pulled up by a hand winch, and the gas samples were secured within seven to ten minutes from the strike, faster in shallower water. The depths were measured by marks on the near vertical and heavily loaded line or wire. The data from very shallow water were obtained from captive fishes that had been living in large wire screen enclosures in the sea for at least one week.

It should be pointed out that in most of our deeper fishes, the swimbladder ruptures when the gas expands on the way up to the surface (Fig. 4). In such an event, the gas escapes into the body cavity of the more or less asphyxiated fish and even out through the connective tissue, resulting in spectacular emphysema such as was graphically illustrated by Ledebur (1936). Unprotected by the swimbladder wall, the gas now undergoes more or less rapid diffusion exchange with the body walls. The rate of these exchanges was determined on 6 specimens of ling and cod that had been caught at 4 atmospheres depth and which were lying on the deck. The nitrogen concentration was found to increase by about one volume per cent per ten minutes—*i.e.*, from 25 to 26 volume per cent. The carbon dioxide increased from 0.5 per cent to 0.6 (0.7) per cent. These determinations were made on the half cc. analyzer (Scholander, 1947) and were accurate to within 0.02 per cent. It is therefore likely that all of our nitrogen figures for fishes from deeper waters are from 1 to 2.5 per cent higher than in the living fish at the bottom. There is no apparent difference between the trawled and hand-lined data.

The following fishes were caught and analyzed.

Fishes from the New England Coast

(See Bigelow and Welsh, 1924)

Brosmius brosme	Cusk
Gadus morrhua	Cod
Fundulus majalis	Killyfish
Melanogrammus aeglefinus	Haddock
Menidia notata	Silverside
Merluccius bilinearis	Silver hake
Peristedion cp. miniatum	Deep sea robin
Pollachius virens	Pollack
Pomolobus pseudoharengus	Alewife
Prionotus carolinus	Sea robin
Sebastes marinus	Rose fish
Stenotomus versicolor	Scup
Tautoga onitis	Tautog
Tautogolabrus adspersus	Cunner
Urophyces chuss and tenuis	Ling

Fishes from the Lerner Marine Lab., Bimini

(See Jordan and Everman, 1896–1900)

Acanthurus bahianus	Tang
Apsilus dentatus *	"Black fish"
Balistes carolinensis	Reticulated ocean tally
Balistes vetula	Queen trigger fish
Calamus bajonado	Porgy
Canthidermis sobaco	Ocean tally
Epinephelus guttatus	Red hind
Epinephelus morio	Red grouper
Epinephelus mystacinus	Black grouper
Epinephelus striatus	Nassau grouper
Haemulon album	Margate
Holocentrus ascensionis	Squirrel fish
Lutianus analis	Mutton snapper
Lutianus buccanella	Red snapper
Lutianus vivanus	Long fin red snapper
Melichtys piceus	Black durgon
Ocyurus chrysurus	Yellow tail
Pomacanthus arcuatus	Black angel
Pomacanthus paru	French angel
Seriola dumerili	Amaco jack
Seriola lalandi	Amber jack

* This species was kindly identified by Dr. C. M. Breder, American Museum of Natural History.

The specified data on all of these fishes are presented in Figures 3, 7, and 8. There are in all 16 northern species with samples from 304 specimens and 21 southern species with samples from 80 specimens.

Analysis of swimbladder gas. Immediately after the fish was brought into the boat, it was held under water in a tub and gas was drawn into a 5 cc. syringe tightened with a little glycerine on the plunger and grease on the needle socket. The needle was withdrawn from the fish under water while slowly ejecting gas so as to prevent any sea water from entering the syringe. A small piece of gum rubber cut out of a ¼ inch sheet was stuck on to the needle tip under the water.

Airtightness was ascertained by pressing on the plunger while holding the syringe under water. The sample was usually analyzed within a few hours and did not change detectably during 24 hours when analyzed to an accuracy of 0.02 per cent. Due to the considerable biological spread in the data and the error from the unavoidable delay in getting the sample, it was soon found that the analyses could be made much quicker and simpler and still sufficiently accurately by using the capillary syringe (Scholander and Roughton, 1942).

The following method is an adaptation of the original blood gas method (Roughton and Scholander, 1943) which should be consulted for details. With a 50 division capillary syringe, CO_2, O_2 and N_2 can be conveniently and quickly analyzed within 0.5 per cent of the true values, as determined by more accurate methods.

Reagents: Acid solution: Dissolve 70 grams of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in 100 cc. water and add 3 gm. citric acid.
CO_2 absorber: Dissolve 70 gm. sodium citrate in 120 cc. water and add 4 gm. sodium hydroxide.
Oxygen absorber: Add 15 gm. pyrogallol to 100 cc. of 20% NaOH in a rubber stoppered bottle and cover with a layer of oil 2 cm. thick. The pyrogallol is dissolved under the oil by stirring with a glass rod.
Acid rinsing solution: Add 1 cc. concentrated sulfuric acid to 500 cc. water and add 10 mgm. potassium permanganate

The reagents are stored as shown in Figure 1.

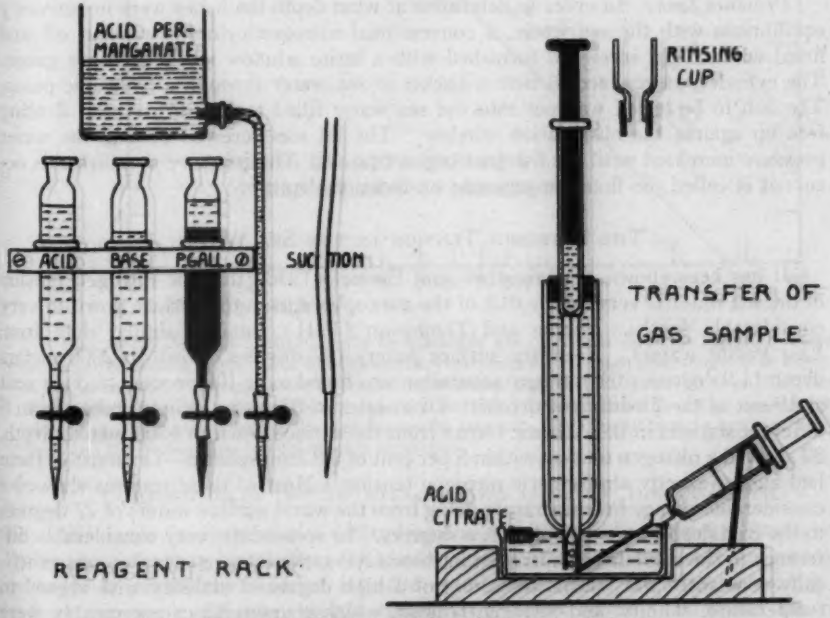

FIGURE 1. Field method for micro gas analysis.

Procedure. The gas sample contained in the 5 cc. syringe is transferred to the capillary syringe by removing the rubber stopper under the saturated acid citrate solution with a forceps and resting the syringe in a wire holder (see Fig. 1). The capillary syringe, with a water jacket, is filled and rinsed with 0.1–0.2 cc. acid citrate and inverted without trapping air over the needle. The first few gas bubbles from the needle are discarded and the few next ones are caught under the cup of the analyzer and immediately drawn into the capillary. The gas solubility in this almost saturated acid citrate solution is so low that no detectable CO_2 losses will occur before the gas gets into the capillary. The solution has good surface properties for micro gas analysis (Scholander and Evans, 1947). The volume of the gas sample is measured in the capillary by drawing the gas bubble down below the fifty mark and slowly adjusting it back to the zero mark. The CO_2 is absorbed by filling the cup with the alkaline citrate solution and slowly drawing it down over the gas bubble in the syringe barrel, where the absorber layers over the acid. After measuring the gas bubble in the capillary, pyrogallol is filled into the cup and similarly layered over the bubble in the syringe and the gas bubble measured in the capillary as before. At the end of the analysis, the fluid is ejected and is removed by suction. A 2 cc. glass or rubber cup is placed on the syringe cup and is filled with the acid rinsing solution. This is drawn down into the syringe and ejected. The washing is repeated until the permanganate color remains, indicating that all the pyrogallol is oxidized. The acid is again ejected and the cup removed. The dead space of the syringe is rinsed twice with acid citrate and 0.1–0.2 cc. left in the barrel for the next analysis.

Pressure tank. In order to determine at what depth the fishes were in buoyancy equilibrium with the sea water, a conventional nitrogen cylinder was cut off and fitted with a tight screw lid furnished with a lucite window and a pressure gauge. The cylinder was connected with a bucket of sea water through a hydraulic pump. The fish to be tested was put into the sea water filled tank with its head floating free up against the observation window. The lid was screwed on and the water pressure increased until the fish just began to sink. The pressure at which this occurred is called the flotation pressure or flotation depth.

The Nitrogen Tension in the Sea Water

It has been shown by Rakestraw and Emmel (1938) that the nitrogen tension of the sea water is very nearly that of the atmosphere from the surface down to very considerable depths. Hamm and Thompson (1941) obtained similar data from East Pacific waters. From the surface waters (17 degrees) down to 2000 meters depth (1.9 degrees) the nitrogen saturation was found to be 100 per cent ± 3 per cent at 19 out of the 21 different depths. Of a series of 85 water samples taken from 6 different stations in the Atlantic Ocean from the surface down to 4500 meters depth, 82 showed a nitrogen tension within 5 per cent of 0.8 atmospheres—*i.e.*, most of them had almost exactly atmospheric nitrogen tension. Most of these stations showed a considerable change in temperature going from the warm surface waters of 27 degrees to the cold deep waters of only a few degrees. In spite of the very considerable difference in the solubility of nitrogen in these layers, the nitrogen tension was practically constant. This seems indicative of a high degree of stability with regard to temperature, salinity, and nitrogen tension, which characteristics presumably were

acquired when these layers were in contact with the surface at some earlier time somewhere. Any subsequent changes in salinity and temperature resulting from mixing of these layers will not change the nitrogen tension of the mixture, because the component layers had the same 0.8 atmosphere nitrogen tension. This, rather than vertical diffusion, may be the reason for the remarkable constancy of the nitrogen tension in the sea water.

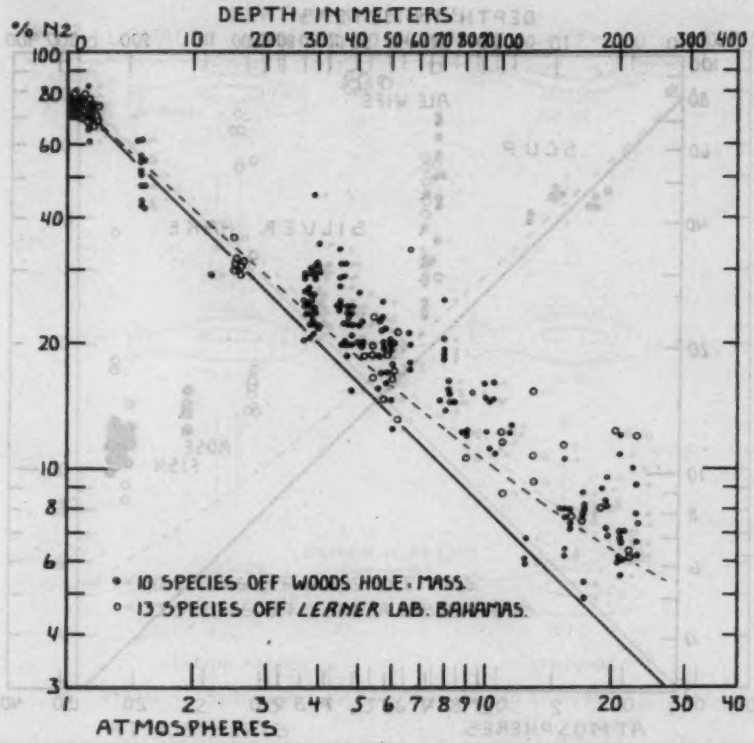

FIGURE 2. Per cent nitrogen in the swimbladder gas of bottom dwelling species in relation to measured bottom depth. The full drawn line represents the nitrogen percentage to be expected if the swimbladder nitrogen were in tension equilibrium with the sea water nitrogen (0.8 atm.). The presented points are uncorrected for post mortem increase known to occur (especially in the deep sea specimens) before the samples can be secured. This amounts to as much as 2½ per cent, and the broken line represents this correction.

COMPOSITION OF THE SWIMBLADDER GAS AS A FUNCTION OF DEPTH

We know from the above determinations that the nitrogen tension throughout the sea water may be taken as 0.8 atmospheres. If we presume that the nitrogen cannot be chemically secreted into the swimbladder, then we would expect it to enter by simple diffusion mostly through the blood stream. Provided the fish

stayed at a constant depth, the nitrogen tension in the swimbladder should, therefore, in time, adjust itself to that of the surrounding water—*i.e.*, 0.8 atmospheres at all depths. If such were the case, however, the nitrogen percentage in the swimbladder gas should come out as 80 divided by the total pressure in atmospheres. At one atmospheres pressure—*i.e.*, at the surface—the nitrogen should be 80 per cent, at 10 atmospheres total pressure the nitrogen should be 8 per cent, at 100

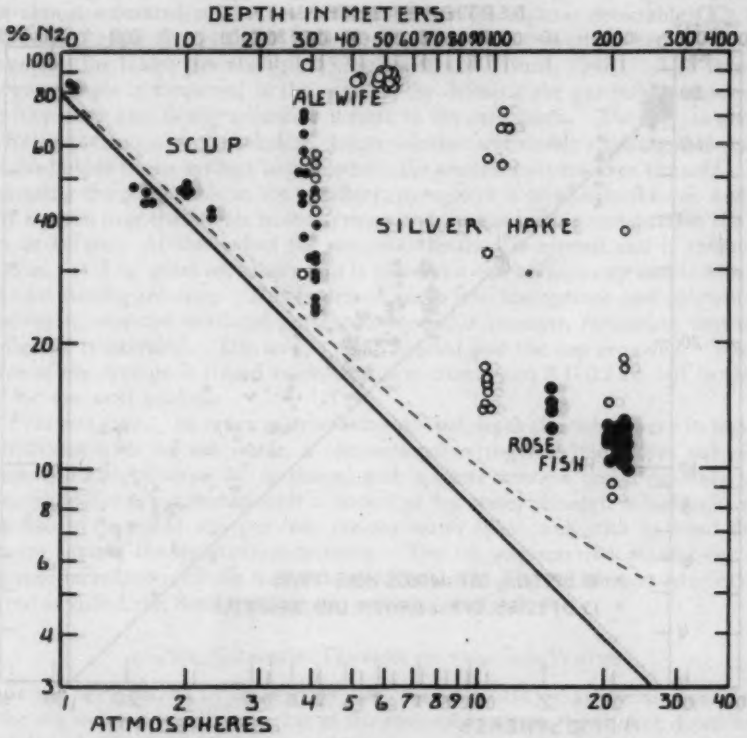

FIGURE 3. Percentage nitrogen in the swimbladder gas of vertically migrating species. The presented points and lines same as Figure 2.

atmospheres 0.8 per cent, etc. If the log. of the depth in atmospheres is plotted against the log. of the nitrogen percentage, we would accordingly get a straight line relation such as shown in Figure 2.

We see in Figure 2 that the predicted correlation between the nitrogen percentage and the depth holds rather well for most of the species. The correlation is even better if we consider that the nitrogen per cent in the intact swimbladder at the bottom was lower than found 5–30 minutes later when the gas was taken from the body cavity of the asphyxiated fish. The dotted line in Figure 2 and Figure 3 indicates how much nearer the points would fit the prediction if the ob-

served values were corrected by subtracting 2.5 volume per cent, or, as is actually done here instead, by adding 2.5 per cent to the predicted line. We may, therefore, conclude that the nitrogen tension in the swimbladder of all those fishes was nearly in equilibrium with that of sea water, and hence is essentially determined by the latter. The CO_2 per cent in all of our samples was rarely above 1 per cent and it is, therefore, clear that the entire pressure work rests on the oxygen secretion (Fig. 6).

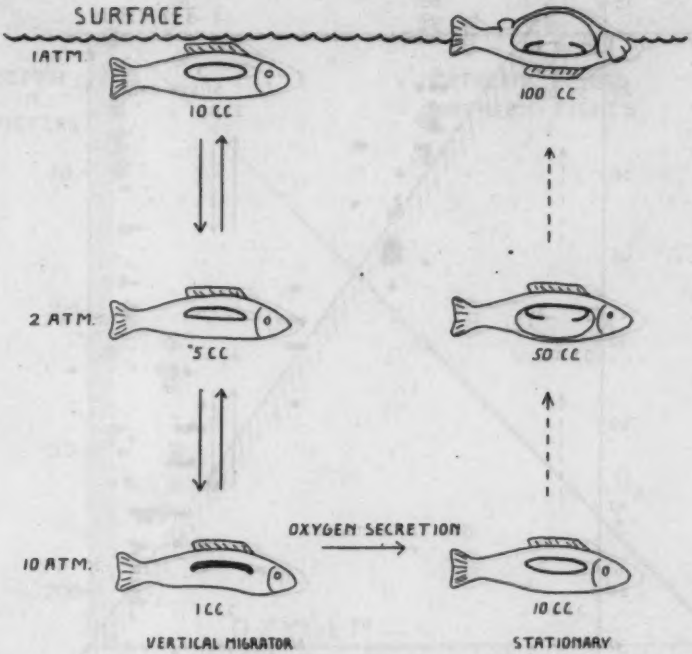

FIGURE 4. Swimbladder gas volume in relation to depth.

The above simple correlation does not hold, however, in all species. The scup, alewife, silver hake, and rose fish are conspicuous exceptions (Fig. 3). In many of these, the nitrogen tension in the swimbladder recalculated to the depth at which they were caught would amount to more than ten atmospheres. How do these high nitrogen tensions come about?

The rose fish is caught at the bottom in many places in great abundance, but when trawled for at night in the same locality it is hardly ever caught. This makes one suspect that they migrate up at night as so many planktonic organisms do. If so, they may acquire a nitrogen percentage characteristic of lesser depth. This depth may conversely be estimated if we know the nitrogen concentration. Corrected for a likely gain of 2 per cent, the nitrogen in our rose fishes caught at about 200 meters depth would be $11 - 2 = 9\%$. This corresponds to a depth of 80/9

= 8.9 atm. or 80 meters. It is likely that our rose fish migrate up to at least 80 meters depth during the night, a figure that corresponds with observed habits of these fishes (see Figure 5).

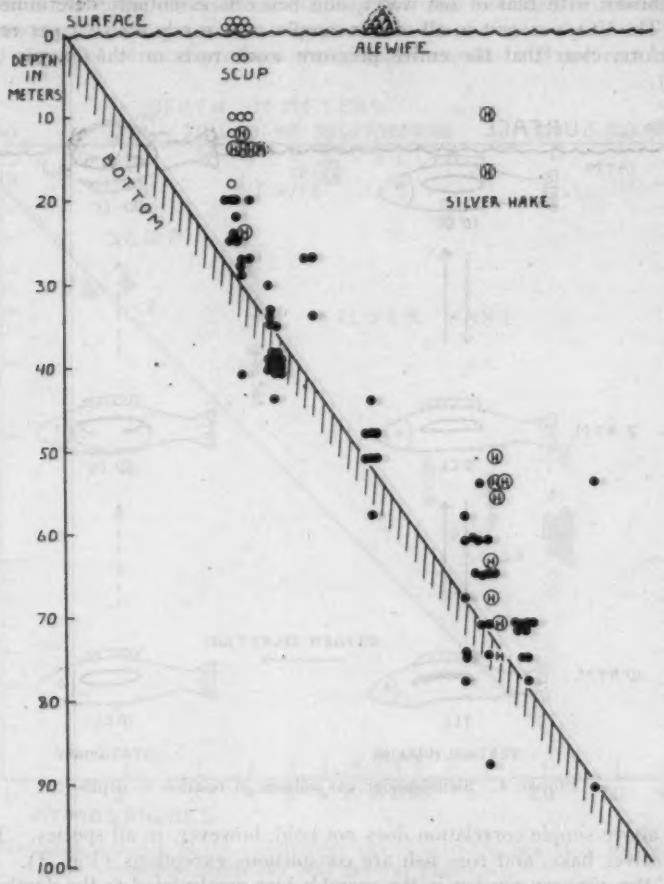

FIGURE 5. Flotation pressure (depth) in relation to bottom depth. Black points are bottom dwelling species: sea robin and ling.

Vertical migration is therefore an obvious reason why fishes can get a seemingly too high nitrogen tension in their swimbladder. We shall see that the amount of gas carried in the swimbladder furnishes other evidence for this.

AMOUNT OF GAS IN THE SWIMBLADDER

It has long been known that an important function of the swimbladder is to hold an amount of gas which is sufficient to keep the fish exactly buoyant with the water

—*i.e.*, which maintains its specific gravity equal to that of the sea water (Moreau, 1874). If the fish changes depth the gas in the swimbladder will expand or compress according to the hydrostatic pressure. Consequently, if it wants to maintain its buoyancy, it must compensate by gas secretion or gas absorption. This it may do, or it may not. In Figure 4, left, we have pictured a fish which requires 10 cc. of gas in the swimbladder to make it buoyant at the surface. This gas will have near 80 per cent nitrogen. If this fish chooses to go deeper, all that happens is

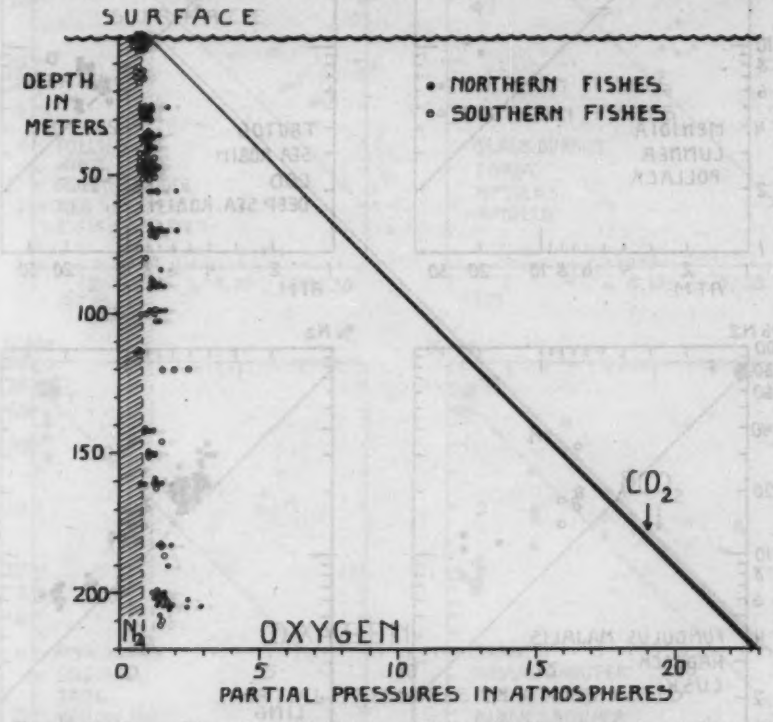

FIGURE 6. Composition of swimbladder gas of bottom dwelling species in relation to depth. The nitrogen values are uncorrected for post mortem increase. With this correction applied, the nitrogen tension would closely approximate the nitrogen tension of sea water, represented by the broken line (0.8 atm.). The CO_2 tension in the swimbladder is represented by the thickness of the line.

that the swimbladder collapses according to the hydrostatic pressure. At 10 atmospheres the original volume of 10 cc. will have shrunk to 1 cc. and the fish will now be 9 grams heavy in the water. If this fish does not, or cannot, secrete any oxygen against this pressure, or if it is caught before it has had time to do so, we will, of course, find near 80 per cent nitrogen in the swimbladder. Recalculated to the depth of 10 atmospheres the nitrogen tension will be 8 atmospheres, and the reason for the high tension is simply that the fish brought the surface gas down. If this

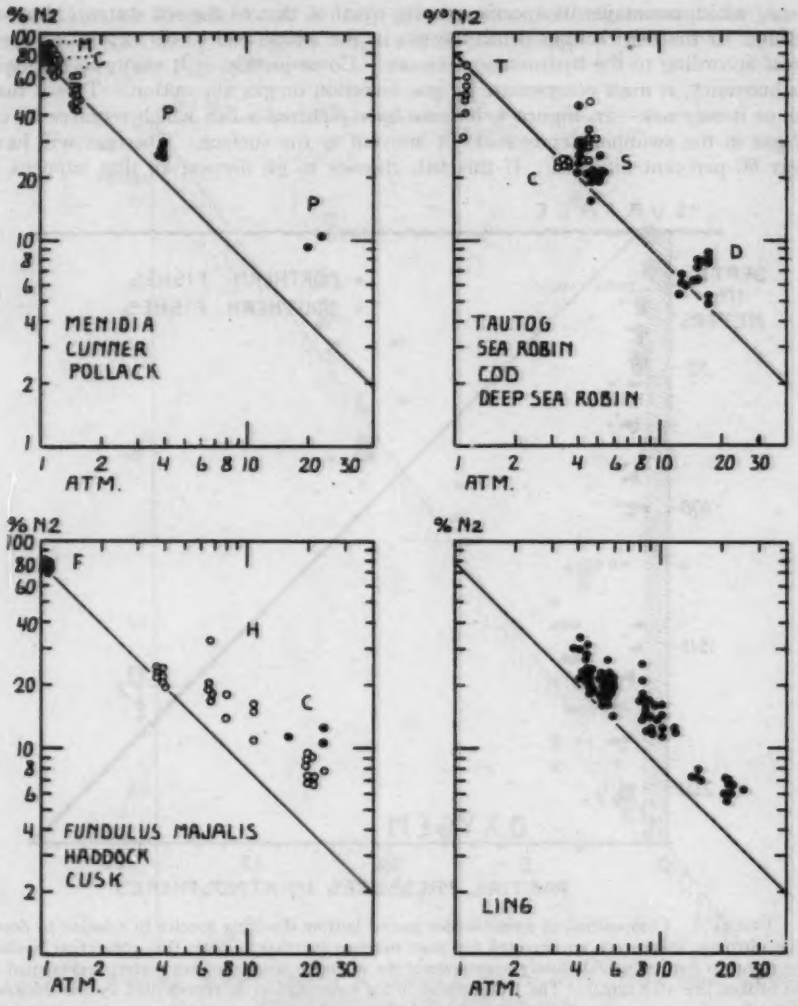

FIGURE 7. Relation of nitrogen percentage in the swimbladder gas to the depth in eleven species of fishes off the New England coast.

fish were tested in the flotation cylinder, it would be found that the fish would be buoyant at the surface and hence had not secreted gas, and from the surface flotation we could hence conclude that the high nitrogen tension was caused by downward migration.

If, on the other hand, the fish is brought down to 10 atmospheres depth and now wants to regain its lost buoyancy, it must secrete 90 cc. oxygen measured at one

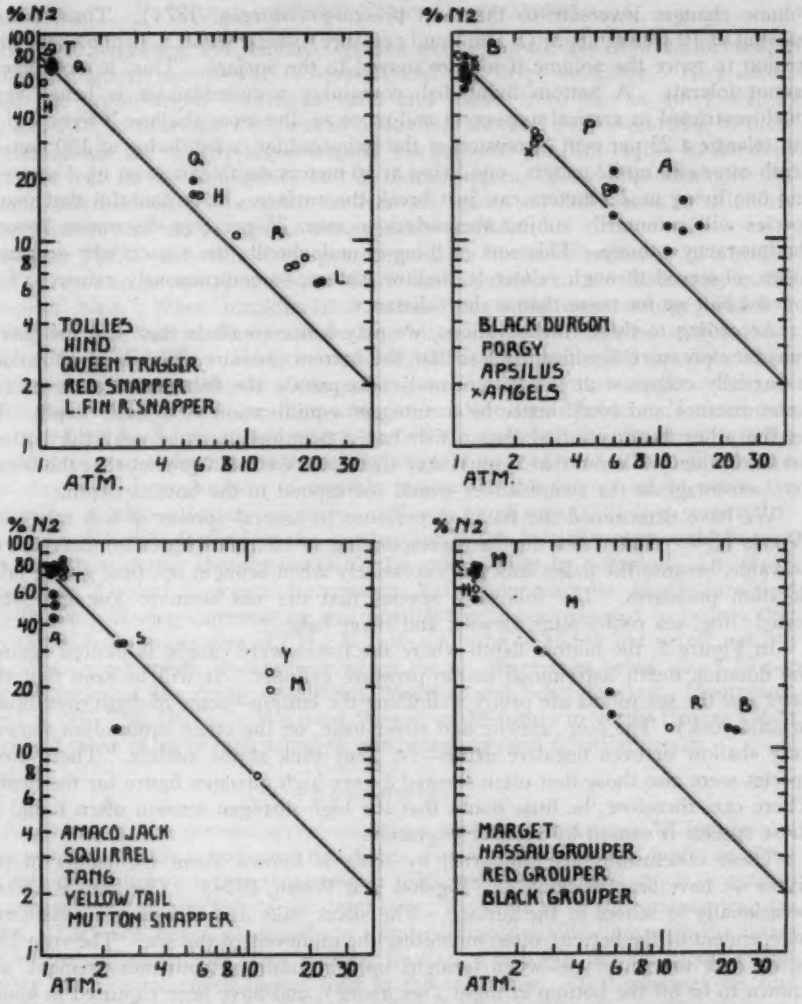

FIGURE 8. Relation of nitrogen percentage in the swimbladder gas to the depth in eighteen species of fishes, taken off the Lerner Marine Laboratory, Bimini, Bahamas.

atmosphere in order to make a total of 10 cc. gas in the swimbladder at 10 atmospheres. This fish is now very much restricted in its upward migration, as any decrease in the hydrostatic pressure will expand the swimbladder gas. Unless the fish has a patent air duct through which to let the gas out (Physostome), it can only very slowly get rid of the gas (Bohr, 1892, 1894).

The expansion of the swimbladder as a function of depth is merely that the

volume changes inversely to the total pressure (Moreau, 1874). Thus, a fish buoyant at 10 meters depth (2 atm. total pressure) would suffer a swimbladder distension to twice the volume if it were moved to the surface. This, it most likely cannot tolerate. A bottom living fish containing a swimbladder is hence very much restricted in vertical movement and more so, the more shallow it lives. If it can tolerate a 25 per cent distension of the swimbladder, a fish living at 100 meters depth can swim up 22 meters; one living at 10 meters depth can swim up 4 meters; and one living at 2.5 meters can just break the surface. It is doubtful that many species will voluntarily subject themselves to even 25 per cent distension beyond the buoyancy volume. This sort of thing is undoubtedly the reason why so many fishes, observed through relatively shallow waters, so conspicuously refuse to follow the bait up for more than a short distance.

According to these considerations, we may hence conclude that, if a fish has a buoyancy pressure significantly less than the bottom pressure, then its swimbladder is partially collapsed at the bottom and consequently the fish can migrate up for some distance and could hence be in nitrogen equilibration at a lesser depth. If, on the other hand, we find that a fish has a flotation pressure near the bottom pressure, then we know that it must stay there and we could predict that the nitrogen percentage in the swimbladder would correspond to the bottom depth.

We have determined the flotation pressure in several species of fish taken off Woods Hole. Data from depths corresponding to 10 atmospheres or more are of no value, because the fishes leak gas excessively when brought up, thus giving false flotation pressures. The following species that did not seem to lose gas were tested: ling, sea robin, scup, alewife, and silver hake.

In Figure 5, the bottom depth where the fishes were caught is plotted against the flotation depth determined in the pressure cylinder. It will be seen that the lings and the sea robins are pretty well along the bottom—some of them even down in solid rock! The scup, alewife and silver hake, on the other hand, often showed very shallow or even negative depth—*i.e.*, they sank at the surface. These three species were also those that often showed a very high nitrogen figure for the depth. There can, therefore, be little doubt that the high nitrogen tension often found in these species is caused by vertical migration.

These conclusions are supported by what is known about the habits of the fishes we have been working on (Bigelow and Welsh, 1924). The scup is known occasionally to school at the surface. The silver hake and alewife are considered independent of the bottom, often inhabiting the midlevels of the sea. The rose fish, which leak too much gas when brought up for flotation depth measurement, are known to be off the bottom at night (see above), and have been captured in abundance on line trawls suspended at 100 to 200 meters over much greater depths (Murray and Hjort, 1912). Ling, cusk, and sea robin are considered strictly bottom fishes.

We have a few compression data on cod, pollack, and haddock from 30 meters depth. They all seemed to leak gas coming up and had shallow flotation depths although rather good nitrogen values. The cod and the pollack are known to live at any level between the bottom and surface; the haddock rarely leaves the bottom (Bigelow and Welsh, 1924).

Notes on Some Early Observations

In 1896, Schloesing and Richard reported analyses of the gas from the airbladder of the Portuguese man-of-war (*Physalia*) and two deep sea fishes. They determined the argon/argon + nitrogen ratio and found it to be: in air 1.184, in *Physalia* 1.18, in *Murenes* 1.85, and in *Synaphobranchus* 1.94. According to these determinations, the relative argon tension was some 60 per cent higher in the deep sea fishes than in the atmosphere and in the sea water. How this seemingly significant difference has come about we can only guess at; differences in solubility and diffusibility of the two gases are probably involved.

Hüfner (1892) determined the gas composition in the swimbladder of a whitefish (*Coregonus acronius*) netted at 60–80 meters depth in the Bodensee, in the European Alps. When breaking the surface the fishes were strongly inflated, floating on their backs; they could be kept alive only when deflated with a sharp stick (v. Siebold, 1858). Hüfner extracted 120 cc. gas from a 300 gm. fish, which would give a flotation depth corresponding well to the bottom depth. Six out of nine fishes had over 99 per cent nitrogen in their swimbladder. If these data are correct, we have here a case where the 6–8 atmospheres nitrogen tension in the swimbladder cannot have been caused by migration downward. It might seem a possibility that decomposition of organic matters at the bottom might cause the production of such gases as hydrogen, methane, or nitrogen. If this occurred to the extent of bubble formation, the gas tension in the bottom water could become high enough to permit these gases to fill the swimbladder by diffusion. Dr. G. E. Hutchinson, Osborn Zoological Laboratory, Yale University, has kindly furnished us with the following comment on this possibility: "I am prepared to believe that very rarely 1–2 atmospheres of CH_4, N_2, etc. might be so added, but surely not 7–8 and certainly not in the Bodensee in which the O_2 never goes below about 75 per cent saturation at any depth (Auerbach, Maerker and Schmalz, Arch. Hydrobiol., Suppl. 3, 597–738, 1924)." We must leave it to the future to verify Hüfner's data or to find other cases of high nitrogen percentage at deep flotation depths.

Conclusions

According to the present survey based on analytical data from 37 species and 384 specimens, it seems indicated that fishes that live at a constant depth, as along the bottom, acquire a nitrogen tension in their swimbladder gas which is near to 0.8 atmospheres irrespective of the depth—*i.e.*, the same as in the surrounding sea water. They are, hence, essentially in simple diffusion equilibrium with the nitrogen in the sea water. In other species, we find a nitrogen tension in the swimbladder that is often much higher than in the sea water. In these cases we find that the flotation depth of the fishes and their known habits, places them at more shallow depth than the bottom so that the high nitrogen tension found in the swimbladder can be satisfactorily explained by vertical migration. We need not think in terms of "nitrogen secretion" in these fishes. The CO_2 tension in all cases was found to be very low so that practically the total gas pressure in the swimbladder above 0.8 atmospheres is made up of oxygen (Fig. 8).

The present work was made possible by the support of the William F. Milton Fund. We are greatly indebted to Dr. A. Baird Hastings and Dr. Eric G. Ball for

helpful advice. We are greatly indebted to Dr. W. F. Royce and Dr. R. J. Buller, U. S. Bureau of Fisheries, Woods Hole, for arranging our participation in the research cruise with M. S. Albatross III and for all the help extended to us on board. Dr. C. M. Breder, Jr., American Museum of Natural History, most helpfully arranged for our work at the Lerner Marine Laboratory, Bimini in the Bahamas. Mr. Michael Lerner gave us the utmost cooperation and free use of his most valuable collection of live fishes. We have had the privilege of cooperation from Dr. I. M. Taylor and Mr. H. H. Woodard who spared no effort in helping us secure preliminary material. Carl Moeller and Johnny Andrews gave most valuable help in constructing our pressure tank.

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ABSTRACTS OF SEMINAR PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

JULY 3, 1951

The effect of stretch on action potentials in muscle. B. LIBET, H. J. RALSTON AND B. FEINSTEIN.

Supramaximal stimulation of the nerve of an isolated nerve-muscle preparation (frog) produces an electromyogram (EMG) which has a greater amplitude in the stretched than in unstretched muscle. The lower EMG of the unstretched muscle rapidly builds up to the full height of the EMG of stretched muscle if the nerve is stimulated repetitively. This increased EMG due to stretch appears to be due to an increase in the number of muscle fibers responding to the maximal nerve volley (*i.e.*, not all neuromuscular junctions transmit in the unstretched muscle), rather than to any increase in size of the action potentials of the individual muscle fibers, because: (a) no increase in EMG occurs with stretch if the muscle (curarized) is directly stimulated instead of the nerve; (b) the twitch tension developed by an unstretched muscle is greater if the muscle is directly and maximally stimulated than if the nerve is so stimulated; (c) partial curarization makes the facilitatory effect of stretch on the EMG become more evident in the monkey. The role of this facilitatory effect of stretch on neuromuscular transmission in the normal animal is not yet clear.

In normal man or monkey, stretch of muscle *decreases* the amplitude of the EMG whether the muscle is activated by maximal voluntary efforts or by supramaximal shocks to the peripheral nerve. Each individual EMG spike also shows broadening and greater complexity in the stretched muscle; this suggests that a greater average desynchronization of the individual action potentials is produced by stretch (in larger muscles). This would cause the reduction in overall EMG amplitude which might be so great as to mask any increase such as is observed in the frog muscles.

Observations on the development of the slime bacterium, Chondromyces crocatus. JOHN TYLER BONNER, CHAMBLESS JOHNSTON AND HAROLD CLIFTON URCHEL.

Chondromyces has in its life cycle an aggregation of bacterial rods which rises into the air to give a small branched fruiting body. The stalk of the fruiting body is composed largely of congealed slime, while the cysts which are borne in clusters at the branch tips enclose large numbers of rods. Upon germination these cysts will split open and liberate the rods which feed and divide, accumulating in larger and larger aggregates until the final fruiting. All these stages were followed with time-lapse motion pictures and numerous novel details concerning their development were revealed. Also some preliminary experiments have been performed on the mechanism which guides the bacteria into the central collection masses and from these it was concluded that the tracks laid down by the previous passage of rods are extremely important in physically orienting the direction of movement of the cells. But also, there is an attraction mechanism which does act at a distance without the help of such tracks. This mechanism could be chemotaxis but there is no positive evidence that such is the case.

An application of ion-exchange resins to the isolation of amino acids from biological material. LEON S. CIERESZKO.[1]

With the aid of the ion-exchange resins Amberlite IR–120 and IRC–50, we have been able to isolate, in good yield, and in pure form, the amino acids from rather complex mixtures in which there is one major amino acid component.

[1] Aided in part by a grant from the Permanent Science Fund, American Academy of Arts and Sciences.

The amino acid was removed quantitatively from the extract or filtered bacterial culture medium by passing the solution through a column of the strong sulfonic acid cation-exchanger Amberlite IR–120. After rinsing the ion-exchange resin with water, the amino acid was brought into solution by displacement with ammonia. Effluent was collected from the time amino acids appeared in the solution. The solution containing ammonia and amino acids from the IR–120 resin was then passed through a column of the weak carboxylic cation-exchange resin Amberlite IRC–50. Amberlite IRC–50 removed the ammonia from the solution, but permitted the amino acids to pass through in solution. The solution from the IRC–50 resin was decolorized if necessary, concentrated by distillation at low pressure, and the amino acid was then crystallized from the concentrate by the addition of alcohol, or, in the case of the dicarboxylic amino acids, by acidification of the solution to a pH value of 2.8–3.0.

Using the method outlined above, we have isolated D-aspartic acid from cultures of *Bacillus subtilis* grown on a synthetic medium containing DL-aspartic acid, asparagine from hot water extracts of tobacco, and glutamic acid, alanine, and methionine from bacterial culture media.

The method as described is not applicable to the isolation of the basic amino acids.

JULY 10, 1951

Status of the chemistry of Cypridina luciferin.[1] H. S. MASON AND E. F. DAVIS.

In this study an attempt was made to assess the homogeneity of the purest concentrates of Cypridina luciferin available and to test a variety of the structures hypothetically assigned to it. The extremely oxygen-sensitive luciferin was subjected to paper partition chromatography in an atmosphere of oxygen-free hydrogen saturated with the vapor of the developing solvent, and the position of the luciferin was determined by means of light emission in the presence of enzyme. No concomitants could be observed within the limits of sensitivity of the tests employed. However, the luciferin itself separated into two substances, both of which served as light-producing substrates for luciferase. The hypotheses which provide that luciferin is a phenol (Anderson, 1936), a phenolic hydroxymethylketone (Chakravorty and Ballentine, 1941), a naphtholic hydroxymethylketone (Spruit, 1946), a flavin (Eymers and van Schouwenburg, 1937; Johnson and Eyring, 1944), or a compound containing labile phosphate (McElroy and Ballentine, 1944) were not confirmable.

Studies on the metabolism of fetal and placental tissues. CLAUDE A. VILLEE AND DOROTHY T. BALZER.

Tissue slices, prepared from fetuses and placentas obtained at therapeutic interruptions of pregnancy, were incubated in Warburg vessels in a Ringer-phosphate medium containing C^{14}-labeled glucose, pyruvate, acetate, oxalacetate or glycerol. Carbohydrate metabolism was studied in the tissue slices and oxygen utilization, substrate utilization, and glycogen, carbon dioxide, and lactic acid production were measured. Fourteen fetuses ranging in age from nine to about twenty-three weeks were used. It is difficult to make comparisons between different experiments, for not only are the fetuses of different ages and genetic stocks, their mothers had different diseases. Yet the metabolism of these tissues is not dissimilar; the tissues from fetuses of diabetic mothers behave as normal, not as diabetic.

It was possible to observe the development of the enzyme glucose-6-phosphatase: it appears at about 11 weeks in the liver and at about 16 weeks in the kidney. Although the fetus has been reported to be insulin-fast, the addition of insulin *in vitro* produces an increase in glucose uptake, glycogen formation and pyruvate and lactate production in diaphragm, heart and kidney, as in the comparable adult tissues.

By as early as the eighth week of development, the fetal tissues are well differentiated biochemically and show the patterns of carbohydrate metabolism characteristic of the corresponding adult tissue. Liver has a high glycolytic rate, brain has the highest rate of glucose utilization

[1] Work supported by the Research Corporation, New York, and the Eugene Higgins Fund of Princeton University.

and the lowest rate of pyruvate utilization of the five tissues studied: brain, heart, diaphragm, liver, and kidney. The heart has the highest rates of pyruvate utilization and lactic acid production. The tissues show a wide variation in initial glycogen content, from 27 mg. per gm. wet weight in liver to 0.16 mg. per gm. in brain.

The diaphragm rapidly metabolizes pyruvate to CO_2, heart metabolizes more pyruvate than glucose to CO_2, and the liver metabolizes about equal amounts of the two. Brain metabolizes pyruvate to CO_2 well but uses acetate only slowly. In contrast, liver metabolizes acetate at least as well as pyruvate. Oxalacetate is decarboxylated rapidly by all tissues and the presence of unlabeled oxalacetate decreases the amount of radiopyruvate metabolized to CO_2. The oxalacetate is apparently decarboxylated to pyruvate and thus dilutes the radiopyruvate present.

Auxin as the hormonal factor normally limiting the differentiation of xylem cells around a wound. W. P. JACOBS.

The hypothesis expressed in the title has been confirmed in the following ways: Auxin shows a strong polarity of movement in Coleus internodes, whether one tests the auxin naturally present or adds synthetic auxin. The strong basipetal movement of auxin is paralleled by a strong basipetal polarity of xylem regeneration; the relatively slight acropetal movement of auxin is paralleled by a slight acropetal differentiation of xylem. Removal of all the leaves and buds below the wound has no effect on xylem regeneration—this is expected since it has been shown that these leaves produce relatively small amounts of auxin and that only a small percentage of the amount produced can move up the stem. Removal of the leaves and buds above the wound markedly reduces the amount of xylem regeneration, the amount of reduction varying with the number of auxin sources removed. A concentration of synthetic auxin such as normally comes from Leaf No. 2, the major auxin source, completely replaces the xylogenic effect of Leaf No. 2. Finally, auxin has been shown to be normally present in very small amounts in Coleus internodes; hence, it can conceivably be acting as a limiting factor.

JULY 17, 1951

Polarographic investigation of the reaction between organic mercury compounds and thiols. REINHOLD BENESCH AND RUTH E. BENESCH.

The importance of thiols in biological systems has led to the wide use of organic mercury compounds for their detection and characterization since these compounds are relatively specific in their interaction with -SH groups, in contrast to divalent mercaptide-forming metals such as cadium or copper.

Polarography provides a specific and sensitive method for the quantitative assay of organically bound mercury, which has been lacking hitherto. This method was therefore used to study the reaction of two classes of organic mercury compounds, *i.e.*, phenylmercuric compounds and mercurated allylamides (used in medicine as diuretics), with mono- and dithiols.

(1) Reactions with Glutathione: The organic mercury compounds investigated form stable mercaptides with glutathione which can be detected and determined polarographically since they are reduced at different potentials from the free mercurials. Moreover, it is possible to determine the glutathione mercaptides and the free mercury compounds in the presence of each other.

(2) Reactions with 2,3-dimercaptopropanol (BAL): The reaction of phenylmercuric compounds with BAL is stoichiometric and leads to the formation of an insoluble dimercaptide. The reaction between the mercurated allylamides and BAL leads first to the formation of an unstable intermediate product. This can be identified as the dimercaptide by working at 0° C., where it is sufficiently stable to permit its polarographic characterization. This product decomposes at room temperature with the formation of an insoluble precipitate. Polarograms of the supernatant solution showed that the mercaptide waves had disappeared completely and free

organic mercury compound had reappeared. The quantitative relationships are in agreement with the following reaction mechanism:

$$CH_2OH \cdot CHSH \cdot CH_2SH + 2\,Hg^{+}RCOO^{-} \rightarrow CH_2OH \cdot CHSHgRCOOH \cdot CH_2SHgRCOOH \rightarrow$$
$$\rightarrow CH_2OH \cdot \underbrace{CHS \cdot CH_2}SHg + Hg^{+}RCOO^{-} + HRCOOH$$

The rate of the progressive reaction is greatly increased by an increase in temperature. It is also accelerated by increasing hydrogen ion concentrations, which is undoubtedly related to the ease with which the C—Hg bond in the mercurated allylamides is split by acid. The conversion of the diuretic molecule into an insoluble cyclic mercaptide by BAL provides an explanation for the suppression of mercurial diuresis by this compound.

Blood and liver lipids in the northern pike, Esox lucius. CHARLES G. WILBER.

Practically all the work on lipids in fish has been restricted to investigations on marine forms. It was therefore decided as part of an overall program of arctic biological research to study the lipids in various Alaskan freshwater fish. The northern pike, Esox lucius, taken from Lake Minchumina was selected as one of these. The blood lipid values in mg. per 100 cc. are: total fatty acids, 771; cholesterol, 183.3; phospholipids, 411.6. The liver lipid values in per cent fresh tissue are: total fatty acids, 9.1; cholesterol, .89; phospholipids, 3.1.

A comparison of these values with those for various marine fish shows that there is appreciably less fat in the liver of the northern pike than in that of many marine fish, where total oil values of as high as 60 per cent are found. Blood fatty acids and cholesterol are not remarkably high, but the phospholipids are in greater concentration than in the blood of various animals such as man, dog, or rabbit.

The phospholipid value in the liver is much like that in the livers of many poikilothermes and of some homothermes. On the other hand the cholesterol concentration of close to 1 per cent in the liver is noteworthy. Whether it is all cholesterol or whether some other sterol is included was not ascertained. It is known, however, from previous work that the chief sterol in fish is cholesterol.

In view of the high liver cholesterol, it is suggested that further intensive studies of the liver of the northern pike be made. A complete fractionation and identification of the sterol complex is indicated. As a practical matter careful attention should be paid to the vitamin content of these livers.

Calcification in molluscs: The localization of Ca^{45} and P^{32} in the mantle and regenerating shell. GERRIT BEVELANDER.

Previous observations have shown that the formation of the shell in molluscs consists in the formation of a protein matrix known as the periostrachum, and subsequently the mineralization of this membrane by calcium carbonate.

The periostrachum is elaborated at the distal part of the mantle. One obvious function which can readily be ascertained is the role it plays in supporting and binding the crystals into various layers or strata which make up the shell. Less obvious, but of considerable interest is the fact that the periostrachum is a fairly complex protein containing several constituents which may play an active role in the mineralization of the shell.

In the course of several experimental procedures we have observed that Ca^{45} and P^{32} are taken up by the mollusc when these substances are placed in water in trace amounts. Subsequent examination of the mollusc reveals the presence of Ca^{45} on the periphery of the mantle. The P^{32} was located on the inner surface of the mantle which we have also identified as a region which is rich in alkaline phosphatase. Radio-autographs of regenerated shell, grown in the presence of Ca^{45} and P^{32} show Ca^{45} to be localized in the crystals of the shell; P^{32} is confined to the protein membrane surrounding the crystals.

Our experiments have demonstrated that calcium and phosphate in ionic form are readily ingested from the water; further, these ions are concentrated in the mantle and various other

organs, and finally both Ca^{45} and P^{32} are eventually deposited in the crystalline and organic parts of the shell respectively.

The localization of phosphatase in the mantle, in the region of the mucus glands containing organic phosphate, suggests a parallelism between this correlation and that found in developing bone. In the case of shell formation, however, the final calcified product is calcium carbonate, while in bone the calcified substance is a phosphate complex (apatite). Whatever role alkaline phosphatase may play in the process of calcification, it appears unlikely that it is concerned with a simple hydrolysis to make available phosphate ions to be incorporated into the mineral component of calcified tissues—either bones, teeth or shells.

JULY 24, 1951

Influence of direct current on the long axis distribution of K, Ca and Na in dog nerve.

SIDNEY SOLOMON AND JULIAN M. TOBIAS.[1]

Earlier work has led us to suspect that ultrastructural changes in nerve occurring as part of the excitation process, whether at stimulating electrodes or as part of the process of self-excitation (conduction), are caused or allowed by particular changes in some critical ion ratio, as K/Ca. We have suggested that due to mobility differences Ca and K may be separated along the nerve long axis by the potential existing between stimulating electrodes and also by the potential existing between a now-active and an about-to-become active locus. If so, then on a measurable scale, one might find that a sufficient and relatively prolonged voltage applied to nerve would move both K and Ca toward a negative region, but would move K there more rapidly thus increasing the local K/Ca ratio, opposite changes occurring anodally. Such polar-peculiar changes in this ratio might lead to alterations in ultrastructure such as dispersion and agglomeration which could be responsible for the trans-surface impedance changes known to accompany excitation.

Therefore, dog sciatic nerves have been excised and then polarized on wick or metallic electrodes for varying times with varying voltages, and have been subsequently sectioned into 5 mm. bits along the long axis. These bits were then analyzed for K, Ca and Na.

It has been found that the K/Ca ratio increases at a cathode and decreases anodally. Sodium moves cathodally as expected, but the evidence suggests that some of the Ca actually moves toward the anode, leading tentatively to the conclusion that at least some of the Ca in nerve is bound to a particle, the whole complex being negatively charged.

Influence of various potassium concentrations on the oxygen consumption of frog nerves.

RUDI J. H. OBERHOLZER.[2]

In order to determine the oxygen uptake by excised frog sciatic nerves, bathing in Ringer's solution containing various potassium concentrations, a flow respirometer has been used. The rates of oxygen consumption thus observed have been compared with the resting rates measured for each nerve bathing in frog Ringer at pH 7.1 and 20° C.

In solutions with 20 *mM* KCl a 5% increase of the oxygen consumption has been observed over a period of 4 hours. In solutions containing 40 *mM* KCl the increase of the resting respiration reached a maximum of +14% in ½ hour, then the oxygen uptake gradually decreased, attaining after 4 hours a new steady level of −10%. In a bathing solution of 120 *mM* KCl, where all sodium has been replaced by potassium, the rate of oxygen consumption showed in the first ¼ hour an average increase of +20%. This short-lasting phase was followed by a period of depressed respiration with an average reduction of −30% from the second hour on. The effects were reversible. These results may explain some contradictory observations reported by previous investigators.

[1] This work has been aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago, and in part by a grant from the United States Public Health Service, Panel on Genetics and Morphology.

[2] Fellow of the Rockefeller Foundation for Medical Research and Public Health.

Reaction of substrates and inhibitors with the surface of acetylcholinesterase. I. B. WILSON. No abstract submitted.

The curare effect and axonal conduction. D. NACHMANSOHN. No abstract submitted.

JULY 31, 1951

Chemotropism in Rhizopus nigricans: The staling reaction. DAVID R. STADLER.

This mold, like many others, exhibits oriented growth away from dense populations toward regions of more sparse growth. This behavior (the "staling reaction") is exhibited when all physical factors in the environment are known to be homogeneous; thus it must be a response to chemical heterogeneity.

The earlier finding has been confirmed that the staling reaction is oriented growth away from a metabolic product of the mold (the "staling substance"), not toward higher concentrations of some nutrient.

Attempts to further substantiate the chemical nature of the staling substance by showing its presence in cell-free filtrates have failed. A culture with a heavy inoculum is allowed to grow; then the mold is filtered off. Spores inoculated adjacent to this staled filtrate grow into it as readily as into fresh medium. Thus the staling substance is very unstable. Other experiments indicate that it is not highly volatile.

Experiments with various aged cultures show that staling substance concentration increases at about the same rate as protoplasmic mass.

Concerning the mechanism of turning, the hypothesis has been tested that tropic growth results from differential growth rate across the hypha caused by unequal distribution of a limiting growth factor produced by the mold itself. Thus the staling substance would itself be a diffusable growth factor causing hyphae to grow faster on the side nearer the heavy population and turn away from it. Checks of linear growth rate in heavy and sparse cultures bear out the hypothesis—molds do grow faster in heavier culture. But tests with the cell-free, staled medium show that this medium, which fails to exhibit active staling substance, still does accelerate growth. Thus the growth factor and the staling substance appear to be separate factors.

A second hypothesis—that the staling substance acts by suppressing cell wall enlargement in its presence—has been substantiated by preliminary experiments.

Observations of the flight mechanism of flies. EDWARD G. BOETTIGER AND E. FURSHPAN. No abstract submitted.

Specificity of steroids which inhibit heart beat.[1] IVOR CORNMAN.

Inhibition of pulsation in roller tube cultures of chick heart and mouse heart by desoxycorticosterone was reported earlier. Progesterone also slowed or stopped most chick heart fragments at 0.01 mg/ml and blocked all pulsation at 0.02 mg/ml within 1–3 hrs. Testosterone at 0.02–0.03 mg/ml. (undissolved crystals present) induced partial inhibition at 12–24 hrs. Estradiol in suspension at 0.03 mg/ml, or as a large excess of crystals in the tube had no effect on pulsation, nor did estrone or dehydroisoandrosterone acetate as an excess of crystals. Prolonged exposure to progesterone at 0.02–0.03 mg/ml damaged the fibroblasts. In the presence of an excess of testosterone crystals, the fibroblasts eventually rounded up, but exposure for several days in tubes heavily sown with estradiol, estrone, or dehydroisoandrosterone acetate crystals had no visible effect on these calls. The soluble steroids desoxycorticosterone glucoside up to 0.1 mg/ml and potassium estrone sulfate up to 0.2 mg/ml did not inhibit the pulse during

[1] Supported by an American Cancer Society institutional grant to the George Washington University Cancer Clinic and by a grant-in-aid from the Washington Heart Association.

2-day exposure. A synthetic progestational non-steroid, 1,2-*bis*-(*p*-aminophenyl)-2-methylpropanone-1-dihydrochloride, perceptibly inhibited pulsation at 0.04 mg/ml and blocked it at 0.1 mg/ml. Within this series, substances with progestational activity were the most effective in stopping heart pulsation, whereas other steroids, whether soluble or not, showed little activity.

AUGUST 7, 1951

The nature of the hemolytic action of dodecyl ammonium chloride. WARNER E. LOVE.

The interaction of a typical surface-active cation, dodecyl ammonium chloride (DACl), and human erythrocytes has been investigated by studies of the type of hemolysis produced by it. DACl produces hemolysis by changing the erythrocyte in such a way that it becomes freely permeable to cations. Evidence for this mechanism of hemolysis is provided by the following experimental results:

a. With a newly developed optical method for the separation of swelling from hemolysis, it has been shown that human erythrocytes in a physiological salt solution swell prior to hemolysis by DACl.

b. In solutions of DACl in 0.3 *M* sucrose the cells shrink to a very small volume before they swell and eventually hemolyze.

c. On the addition of a small amount of NaCl to a suspension of such cells in sucrose, they at first shrink very rapidly and then recover their volume more slowly, *i.e.*, NaCl behaves as a typical penetrating substance.

d. DACl increases to a remarkable degree the permeability of erythrocytes to normally non-penetrating non-electrolytes.

Like surface-active anions, this cationic substance protects erythrocytes from its own hemolytic action. Conditions have been found where desorption of the agent from the cell by dilution of the medium with 0.9% NaCl or by changing the pH toward acidity causes immediate and rapid hemolysis.

The effects of both the ionic strength of the medium and the time of exposure to the agent prior to desorption have been studied. Increasing the ionic strength of the medium and increasing the time of exposure both favor the protective action of DACl; it is therefore proposed that something akin to micelle formation occurs at the surface of the cell, the result of which is either to prevent the escape of hemoglobin or to reduce the degree of induced permeability to cations, or both.

The relation between hemolysis and the potassium content of red cells. A. K. AND E. R. PARPART AND T. DEY.

The standard curve of osmotic resistance of the red cells of normal rabbits is very reproducible from animal to animal. The red cells go from no hemolysis at 0.6% NaCl (phosphate buffered in all cases) to complete hemolysis at 0.4% NaCl. Red cells of rabbits were exposed to tonicities in this range at dilutions of 1 to 40. After 1 hour equilibration the environment of the cells was made isotonic with NaCl, and the unhemolysed cells were packed in an air turbine. These packed cells were analysed for K and Na. Over the hemolysis range from 0 to 85% the K and Na content of the unhemolysed cells was found to be the same.

It is necessary to point out that microscopic examination of these unhemolysed red cells reverted to their original volume showed no "ghosts" among the packed cells. This was not true for the unhemolysed cells which were packed in the swollen state.

It is concluded that the osmotic, pre-hemolytic swelling of red cells does not involve a loss of K or a gain of Na by these cells, over 1 hour of exposure to the hypotonic environment. It is further concluded that at least 85% of the red cells in a given population have practically identical K content, and it is suspected that this is true of the entire population.

Some microchemical reactions of the erythrocyte. M. H. JACOBS.

Erythrocytes are chemical systems of very constant composition whose constituents are measured in millionths of a millionth of a gram. As such they can be employed for a variety of chemical tests. Cases have been described elsewhere in which the erythrocytes of certain species show a special sensitivity to silver and to copper, respectively, and can be used to detect traces of these elements.

Further uses of erythrocytes as microchemical reagents are provided by a group of reactions in which swelling and hemolysis in solutions of NH_4Cl give visible evidence of the presence of various specific substances in low concentrations in the surrounding medium.

By taking advantage of the "catalysis" of the entrance of NH_4Cl by bicarbonates it is possible to estimate very small quantities of the latter and also carbon dioxide tensions. In the presence of known amounts of bicarbonates, means are provided for the assay of the enzyme carbonic anhydrase and its inhibitors. Low concentrations of so-called indifferent narcotics can be estimated by their ability to retard NH_4Cl hemolysis. An even stronger inhibitor is tannic acid. Very small quantities of it and of substances which neutralize its inhibiting effect such as organic bases and proteins can likewise be measured by the NH_4Cl hemolysis method.

A method of possible practical usefulness for the estimation of urinary ammonia is provided by the characteristic swelling of erythrocytes exposed to ammonium salts in a suddenly alkalinized medium. By photoelectric-photographic recording, concentrations of the order of 1×10^{-5} *M* can be measured in this way. Less delicate, but practically useful, are other methods employing an ordinary photoelectric colorimeter or even no equipment but common laboratory glassware and the naked eye.

AUGUST 14, 1951

The occurrence of twinning in Fundulus heteroclitus after centrifugation. J. R. SHAVER.[1]

Twinning occurs in *Fundulus heteroclitus* following centrifugation of eggs for 30 mins. × 293 g, beginning 10 to 30 minutes after fertilization. This observation was confirmed, twinning occurring in 0.75% of eggs centrifuged under these conditions. Two hundred eggs were isolated immediately following treatment, and the complete developmental history of two cases of twinning was described; both were cases of complete twins (no fusion of main axes), but cases of anterior duplication and "parasitic" twins have also been observed. From 37.3 to 72.8% of eggs centrifuged during the period of blastoderm formation failed to develop embryos, and 18–52% of the survivors displayed abnormalities. Embryos with skewed axis were the commonest abnormality found (29.7% of abnormal isolates). Deficiencies in mesodermal structures (no somites, reduced number of somites, unilateral somite deficiency, reduced trunk) characterized many embryos; it is thought that these deficiencies were due to irregularities of the germ-ring seen in many of the isolated embryos. Embryos centrifuged in later stages (2-cell, many-cell, gastrula, early embryo), although displaying characteristic "skewing" of cleavage pattern or embryonic axis, did not develop into twins, and the percentage of normal embryos resulting approached that of the controls. It was suggested that in Fundulus the period of formation of the blastodisc is especially sensitive to disorientation of materials that might represent a "primary organization center"; if it is assumed that cortical (or subcortical) materials which form the blastodisc early establish physical contact and rates of reaction with underlying substrata, necessary for normal embryogenesis, the results of the centrifugation might be interpreted as disturbing these relationships.

Significance of the periblast in teleost gastrulation. J. P. TRINKAUS. No abstract submitted.

[1] Aided by Summer Research Professorship, University Research Council, University of Missouri.

Specific fertilizin agglutination of dead sperm. CHARLES B. METZ AND JOANNE DONOVAN.

Properly prepared dead sperm should be useful for the study of the role of the sperm surface in fertilization. Ideally the sperm should be killed and fixed without appreciable alteration of the specific molecular organization of the cell surface.

To prepare such material washed living sperm were treated with the killing agent and when necessary washed again by centrifugation or dialysis. The dead sperm were then tested for agglutination with homologous fertilizin. Since fertilizin agglutination is a highly specific reaction, this test should provide a fair index of the effect of the killing agent on at least one sperm surface group.

Arbacia sperm, which were examined in most detail, failed to give satisfactory agglutination after killing with most agents. However, sperm treated with alcoholic Bouin's fixative (equal volumes of sperm and 25% to 50% fixative) agglutinated strongly on addition of fertilizin. Furthermore, the fixed sperm paralleled living sperm with respect to agglutination by a number of other agents (Asterias egg water; Nereis, Echinarachnius, Mactra fertilizins; Arbacia and Asterias bloods; heparin; univalent Arbacia fertilizin). Fixed and living Arbacia sperm differed in ability to agglutinate in Mactra tissue fluid. However, the dead sperm agglutinin in this material was absorbed by living sperm. Thus the reactive groups for the Mactra agent are present on both living and fixed sperm. Apparently, then, Arbacia sperm retains its specific surface organization to a very considerable degree when treated with Bouin's fixative.

Experiments to determine if amino acid side groups are essential for dead sperm to agglutinate with fertilizin indicate that phenolic and sulfhydryl groups are not essential but suggest that amino groups must be free at the sperm surface for the sperm to agglutinate.

AUGUST 21, 1951

Cooling as a stimulus to smooth muscles. JOHN F. PERKINS, JR. AND WILLIAM H. JOHNSON.

We have quantitatively studied the effects of rapid and slow cooling alone as a stimulus causing contraction of various mammalian smooth muscles and of frog intestine when cooled below 10° C. Rapid and slow cooling act as graded stimuli, rapid cooling being the more effective, as noted by Starling in 1912.

When degrees of rapid cooling are plotted against heights of isotonic contraction, one obtains an S-shaped "cooling-response" curve which falls off with extreme amounts of cooling. That this falling off is not due solely to an increased "viscosity" or internal mechanical resistance preventing shortening is shown by the fact that the same degree of falling off occurs with isometric recording. It appears more likely, then, to assume that with extreme cooling less energy is released, either for shortening, or for developing tension.

Several lines of evidence indicate that cooling alone acts on a different system than epinephrine. For example, after Dibenamine, which reverses the action of epinephrine so that the latter now tends to produce lengthening, cooling still causes a contraction. Normally, the separate effects of cooling and of epinephrine are additive when both are applied simultaneously, but after Dibenamine the two stimuli tend to cancel (algebraic addition, epinephrine having minus sign).

Recent studies indicate that increased amounts of potassium ion potentiate the action of cooling alone as a stimulus, shifting the cooling-response curve to the left. Whether cooling alone will act as a stimulus to the smooth muscles of marine fauna, with and without additional potassium, is being investigated.

It is hoped that an assault upon the excitation-conduction-contraction systems in smooth muscles may be facilitated by using cooling alone as a stimulus, in conjunction with a method developed by us (1950) for studying the viscous-elastic properties of smooth muscles.

Studies in carbohydrate and lipid metabolism, using C^{14}-labelled glycerol. M. L. KARNOVSKY.[1]

Radio-glycerol should be a useful tool in the elucidation of the pathways of lipid metabolism—especially in studies on the biosynthesis of phosphatides. Since glycerol may be considered an offshoot of the glycolytic cycle, the use of radio-glycerol should yield interesting data on lipid-carbohydrate inter-relationships.

C^{14}-labelled glycerol has been synthesized, and metabolic experiments carried out on rats. The labelled compound was administered in normal saline, by intraperitoneal or intravenous injection, or by intragastric intubation, and the animal was immediately placed in a metabolism train. The expired CO_2 reached its peak activity in about 70 to 90 minutes, and the curves were apparently independent of the mode of administration of the glycerol. After 6 hours the animal was sacrificed, and the organ lipid and carbohydrate fractions examined. About 50% of the administered counts had been expired, and about 5% of the administered counts were to be found in the liver lipids. The phosphatides were significantly more active than the neutral fats.

In one series of experiments animals were dispatched at different times after administration of the radio-glycerol. The blood sugar and liver-glycogen reached peak activity at about one hour, and at this peak the amount of liver glycogen derived from glycerol was about 15–20%. The amount of administered glycerol that had then gone to glycogen was about 5%.

Experiments have also been carried out with triglycerides labelled in the glycerol portion of the molecule. The most striking feature has been the decrease in the ratio of the activity of the phosphatides to that of the triglycerides in the case of triolein. Tributyrin behaved essentially like free glycerol.

A number of studies have been carried out *in vitro*, in which rat liver slices have been presented with radio-glycerol, sometimes together with non-radioactive pyruvate. These experiments, together with their converse experiments, have led to some interesting data on the behaviour of the DPN-linked glycerol-dihydroxyacetone : pyruvate-lactate system.

A selective mechanism in Escherichia coli. LILLIAN K. SCHNEIDER AND HELEN CROSS.

Cultures of a histidineless mutant (h−) of the bacterium *Escherichia coli*, serially transferred for long periods of time in medium containing optimum histidine, show a gradual increase in the number of histidine independent (h+) cells, until an equilibrium ratio of 1.3 h+ per 10^8 h− is reached. This ratio has been maintained in one experiment for more than 3100 generations. Mutation pressure alone is not sufficient to account for this equilibrium level since the mutational equilibrium, predicted from independent measurements of the forward and back mutation rates, is 1 h+ per 100 h−. Specific selection against h+ was postulated.

The periodic replacement of the entire population by mutations conferring a selective advantage upon the bacteria in which these changes occur has offered an explanation of the specific equilibrium reached. When a mutation to a fitter type occurs, all other mutant types that have arisen in the population are simultaneously eliminated. Since in our experiments there are from 10^4 to 10^6 times as many h− as h+, the advantage mutant occurs in the h− component first. This fitter h− now becomes predominant in the population and eliminates the previous h− and h+, resulting in an apparent specific selection against h+.

At least two of the steps to successively advantageous mutations are due to intrinsic growth rate differences in the medium in which selection has occurred, suggesting that the growth rate difference is medium-dependent. However, this explanation does not hold in all cases and some steps may involve interactions between different components in the population.

Periodic selection thus could act to maintain the stability of a bacterial population in an environment to which it is suited. At the same time it acts to preserve the bulk of the genotype while allowing for variability in a new environment.

[1] All the experiments described were carried out with the collaboration of Mr. L. I. Gidez and Dr. C. T. Teng.

GENERAL SCIENTIFIC MEETINGS

AUGUST 27–28, 1951

Drugs inhibiting growth and nucleic acid synthesis in Arbacia embryos. DOROTHY T. BALZER AND CLAUDE A. VILLEE.

Mathews (1904) and Sollman (1906) had shown that development was retarded by atropine and pilocarpine, and previous work (Villee *et al.*, 1949) had shown that dinitrophenol, malononitrile, uranyl nitrate and nitrogen mustard, which inhibit cleavage, also inhibit the synthesis of ribonucleic acid (RNA), desoxyribonucleic acid (DNA), or both. The effects of atropine, acetyl choline, eserine and pilocarpine on development and on phosphorus metabolism in developing Arbacia eggs were studied using radiophosphorus. Embryos were incubated 7½ hours in 100 ml. of sea water containing the drug and 3 to 5μC of P^{32}, then homogenized in ice-cold 7% trichloracetic acid and fractionated as in the previous (1949) experiments.

All drugs tested showed an acceleration at low concentrations, 5 mg. per 100 ml., and an inhibition at higher concentrations, 60, 100, or 500 mg. per 100 ml., of growth and phosphorus metabolism, in the order eserine > pilocarpine > atropine > acetyl choline. Eserine affects the enzymes responsible for the uptake of phosphorus into the acid-soluble P fraction of the cell; it has little or no effect on phospholipid P metabolism. Its most marked effect, the one most closely correlated with the effects on cleavage, was its inhibition of the incorporation of P^{32} into the DNA P fraction. These experiments do not indicate whether the action of eserine is on some part of the cleavage mechanism and DNA synthesis is inhibited secondarily, or whether the eserine acts on some phase of DNA synthesis and cleavage is secondarily retarded for lack of DNA. The metabolism of RNA phosphorus is unaffected, even by 100 mg. of eserine per 100 ml., at which concentration DNA P incorporation is only 16 per cent of the control value. Pilocarpine also showed a marked inhibition of the incorporation of P^{32} into DNA but no inhibition of RNA metabolism.

A new DNA fraction of the cell nucleus. JAY BARTON II. See abstract in "Reports on Lalor Fellowship Research."

Hemolysis of sheep erythrocytes by organic mercury compounds. RUTH E. BENESCH AND REINHOLD BENESCH.

Organic mercury compounds of the phenylmercuric and of the mercurated allylamide type have been found to be actively hemolytic towards sheep red cells. Two per cent suspensions of sheep erythrocytes were mixed with the organic mercury compounds under isotonic conditions and shaken gently at 37° C. Aliquots of the suspension were removed at definite time intervals. The intact cells were separated immediately by centrifugation and the extent of hemolysis was measured by colorimetric determination of the hemoglobin in the supernatant fluid. The phenylmercuric compounds caused hemolysis in lower concentrations than the mercurated allylamides. Thus half the cells were lysed in 28 minutes by 5×10^{-4} *M* phenylmercuric hydroxide, while it took 100 minutes to lyse half the cells with 8×10^{-4} *M* Mersalyl. The rate of hemolysis was found to vary enormously with slight changes in the concentration of both types of organic mercury compound. It was further demonstrated that interaction of the organic mercury compounds with an equimolar amount of glutathione, which results in the formation of organic mercury mercaptides, abolished the hemolytic effect of both types of organic mercury compound. These findings suggest that the lytic properties of these compounds are based on their interaction with —SH groups essential for the maintenance of the intact cell structure. Since it has been shown that the red cells of certain fish are lysed by extremely minute concentrations of silver and inorganic mercury ions, it is planned to investigate the effect of organic mercurials on such cells and to correlate it with the concentration of —SH groups in these cells.

Propagated changes in light scattering accompanying activity in nerve.[1] SHIRLEY H. BRYANT AND JULIAN M. TOBIAS.

Little or nothing is known of the changes in protoplasmic ultrastructure (molecular-macromolecular) which may accompany activity in nerve. Toward the detection, measurement and understanding of such components of function, experiments have been designed which yield measurements of changes in light scattering by nerve which accompany impulse propagation. To date, many of our findings have been similar to those of Hill (1950). However, in addition we have found changes in active frog nerve, and the temporal course of the changes seen has been much different in our experiments as compared to those of Hill.

The white light scattered at right angles to an incident beam impinging on a 2 mm. length of frog, crustacean or squid multi-fibered nerve or squid giant axone has been measured using a balanced photocell arrangement.

In essence, what one observes is that the passage of a burst of impulses is accompanied, in some cases, by an initial, small, short-lived increase in scattering. More constantly, with or without this initial increase, there is a more delayed, longer lasting and larger decrease in scattering which, in turn, is followed by recovery more or less to the resting condition. In the frog sciatic only the decreased scattering phase has been observed. Conditions upon which the type and duration of changes seen in the other nerves depend are in process of being worked out. However, hypotonicity of the medium has been found to favor the appearance and duration of the early phase of increased scattering.

Failure of the changes to appear with just sub-threshold stimulation or with supra-maximal stimulation in non-conducting nerves, and lack of dependence on electrode orientation and distance of stimulating electrodes from the light region, rule out current spread from the electrodes as the causative agent.

Naively dividing the maximal change in total scattered light by the number of impulses shows, for the decreased scattering phase, a change of from 0.000006% in the frog to one of 0.01% in the crustacea, per impulse.

Experiments to determine the precise time course and meaning of the changes are in progress.

Autogamy of Paramecium aurelia (stock 51.7) grown on non-living media. W. D. BURBANCK AND MADELINE P. BURBANCK.

Experimental animals were obtained from Dr. T. M. Sonneborn. These were grown on the non-spore-forming bacterium, *Aerobacter aerogenes*, grown on lettuce infusion and in the "two anlagen" stage of autogamy were washed free of bacteria according to Parpart's technique. Washed animals were placed individually on a non-living medium consisting of a 1.5 cc. culture made up of 1.0 cc. *A. aerogenes*-lettuce infusion (with the bacteria killed by exposure to 60° C. for 10 minutes) and 0.5% yeast autolysate (original Basmin-Busch by Anheuser-Busch). Following the first post-zygotic fission, one of the progeny was used to start a caryonide. In 24 hours, eight animals were produced. They were each placed in 1.5 cc. cultures of the following media with the indicated results:[2]

(1) Control grown on living *A. aerogenes* went into autogamy on average of 4.5 days.

(2) *A. aerogenes* used as food was killed by exposure to 60° C. for 10 minutes. Animal placed on this medium died in 24 hours with macronuclear disintegration.

(3) Like (2) but 0.5 cc. of 0.5% yeast autolysate added. Progeny of this animal regularly (seven runs) went into autogamy in 4.5 days.

(4) Bacteria killed by autoclaving (15 lbs., 15 minutes) and 0.5 cc. of yeast autolysate added. Some animals showed macronuclear fragmentation or hemixis.

[1] This work has been aided in part by a grant from the United States Public Health Service, Panel on Morphology and Genetics, and in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[2] *Aerobacter aerogenes*, living or killed, used in all eight media. In first four, it was grown on lettuce infusion and on "Cerophyl" infusion for last four. Except for (2) and (5), all *P. aurelia* progeny produced each day were transferred until 64 were obtained and these were then allowed to reproduce without further transfers.

(5) Like (1) but went into autogamy in 4.0 days. This was the Cerophyl control.
(6) Like (2).
(7) Like (3) but less regular, animals went into autogamy twice in five runs.
(8) Like (4).

Heat death in Phascolosoma. ALFRED B. CHAET.

Heat death, according to the "toxic factor" theory, is the result of a toxic substance which is first found at the site of the burn and is later dispersed throughout the animal's body. Although there have been many studies on heat death and the effect of burns on vertebrates, very little work has been done on the lower animals. The present work was undertaken in an attempt to determine whether or not similar toxic substances could be found in heated invertebrates. For this study an unsegmented annelid, *Phascolosoma gouldi*, was chosen. Heating the posterior half of Phascolosoma (for 90 seconds in 76° C. sea water) causes death in about 16 hours. The animal's life is prolonged for approximately 73 hours by ligating the animal before scalding, thereby restricting the major part of the toxic factor to the posterior end of the worm. Additional evidence for the presence of a toxic factor is given by an experiment in which cell-free coelomic fluid is taken from scalded animals and injected into healthy animals. Such an injection causes death within 24 hours, whereas injections of fluids from normal animals have no effect. The toxic factor is heat stable as well as non-dialyzable. It is very soluble in water, slightly soluble in absolute methyl alcohol, but not soluble in ether. Although the toxic coelomic fluid has a lower pH than the normal, the toxic effect is not lost when the pH is readjusted to that of the normal coelomic fluid. By equalizing the potassium content of the toxic and normal fluids, it can be shown that the toxic effects described here are not due to potassium. Preparations of the clam heart, *Venus mercenaria*, have been successfully employed as test material for the toxic substance.

The physical state of the axoplasm in situ in the nerve of the squid mantle. ROBERT CHAMBERS AND CHIEN-YUAN KAO.

It is already known that the axoplasm of the giant nerve of the squid possesses, under normal conditions, a gel-like rigidity which, under both dark and light field illumination, appears glass-clear and structureless. Evidence of a linearly arranged ultra-structure has been inferred from the fact that drops of inert oils and air bubbles, when microinjected *in situ* within the neurilemma, assume ovoid shapes. Tests were made to ascertain the degree of rigidity of the axoplasmic gel within various regions of the axon from its center to the periphery. It was found that aqueous solutions of acid dyes which diffuse readily when injected give definite evidence of a relatively fluid core. When microinjected, the solution takes a preferred route of running along the central core through the nerve. The dye then diffuses radially until the entire axoplasm becomes uniformly colored. When oil or air is injected the ovoid shapes assumed differ according to the site injected. At the center the drops are broad oval. As the site of injection approaches the periphery the drops become narrower until, in the extreme cortical zones, the drops are narrow cylinders. The ends of the drops are always ovoid. All these regions are readily liquefied by the introduction of $CaCl_2$ in concentrations of this salt in sea water. Axons, extirpated from the squid mantle with the cut ends tied off with silk threads, give, when immersed in sea water, no indication of the calcium liquefying action except at the bases of the open cut ends of side branches. All these regions are readily liquefied by the introduction of $CaCl_2$ in concentrations of this salt in sea water.

The internal hydrostatic pressure of the unfertilized activated Fundulus egg exposed to various experimental conditions. ROBERT CHAMBERS, EDWARD L. CHAMBERS AND CHIEN-YUAN KAO.

Eggs which had reached the constant internal hydrostatic pressure of 150 mm. Hg were immersed in varying concentrations of sea water. When the external medium was hypotonic to sea water the pressure rose within three minutes to relatively high values and then almost imme-

diately fell to the original pressure of 150 mm. Hg. A similar change but in the reverse direction occurred when the external medium was hypertonic to sea water.

These experiments indicate a rapid passage of electrolytes through the chorion with the establishment of equilibrium conditions between the electrolytes of the external medium and those of the perivitelline space. To account for the pressure which is maintained at the same value irrespective of the electrolytic concentration of the external medium there is to be inferred the existence of a non-diffusible substance which remains constant in the completely formed perivitelline space. Fertilized eggs in which the blastodisc had completed its formation were freed of the enveloping chorion. The isolated egg cells exhibited no change in diameter irrespective of the concentration of the sea water in which they were immersed. This indicates either that the electrolytes in the yolked egg are combined so as to be osmotically inactive, which is improbable, or that the external surface of the egg, at least in this particular stage, is impermeable to water and to electrolytes.

Behavior of cortical granules in methylated xanthine effects. RALPH HOLT CHENEY.

It is accepted generally that there is a critically essential relationship between the normalcy of the clear, vacuolar-like, cortical granules of the Arbacia egg and normal fertilization. Certain concentrations of di- and trimethylxanthines interfere with the fertilization and development of *A. punctulata.*

To investigate the effect of these compounds upon cortical granules, eggs were pretreated in various molarities of caffeine, theobromine, and theophylline in sea water for different time intervals at the temperature of running sea water (22° C.). Examination of fertilization sequences were made by adding sperm to a hanging drop culture via a micropipette and the visible changes observed continuously at 1100 × under light or dark field illumination until the fertilization membrane formed and was freed completely from the egg surface. Time events were recorded with a stop watch. Normal and centrifuged eggs were employed.

Fertilization was successful and time relationships remained equivalent to the controls as long as the concentration of methylated xanthines, or the immersion period, was insufficient to cause a change in the clear cortical granules prior to the introduction of sperm. Non-fertilization was predictable when the fine, uniform distribution of the cortical granules was seen to be interrupted in part by the apparent disappearance of some of the granules and an irregular grouping into several masses of the majority of these granules.

This change in the physico-chemical behavior of the granules under the influence of methylated xanthines corresponded synchronously with the inability of the egg to be fertilized. The nature of the effect of all three compounds was identical. The intensity of the effect was one of degree in the order of decreasing effectiveness C > Tb > Tp for equivalent molarities.

Urea adduct formation as a means of separating components of lipid mixtures. LEON S. CIERESZKO, M. L. KARNOVSKY AND ANN BRUMM.

Bengen discovered in 1940 that hydrocarbons and their derivatives form crystalline adducts with urea. The structural requirement for urea adduct formation is a sufficiently long carbon chain with little or no branching. The urea adducts are insoluble in organic solvents, and may be decomposed by treatment with water.

Urea adduct formation has been applied to the separation of fatty acids, but not to the fractionation of the non-saponifiable matter of fats.

We have found that cholesterol and squalene do not form stable urea adducts, while glyceryl ethers, such as batyl alcohol, do. Material containing 90–100% of glyceryl ethers has been recovered from the urea adducts obtained from the non-saponifiable matter of shark liver oil, and of the pyloric caeca fat of starfish. Saturated glyceryl ethers may be separated from the unsaturated glyceryl ethers as the saturated compounds react with urea preferentially.

Enzymes of a ribose-forming pathway during the early embryonic development of Chaetopterus. SEYMOUR S. COHEN. See abstract in "Reports on Lalor Fellowship Research."

Alleviation of the anti-mitotic effect of rotenone by alpha tocopherol.[1] IVOR CORNMAN AND EDWARD F. ROGERS.

To analyze the nature of inhibition of Arbacia egg division by rotenone and similar compounds, chemically related naturally occurring compounds were tested for antagonistic effects. Rutin, folic acid and isorhamnetin were without effect on cleavage delay or suppression induced by rotenone or tephrosin. Alpha tocopherol at 0.01 ml/L partly or completely reversed the rotenone effects. A delay of 65 minutes induced by 0.004 mg/L rotenone was reduced to ten minutes and the ten minute delay resulting from exposure to 0.002 mg/L was abolished by alpha tocopherol. Oils which lack vitamin E activity (mineral oil and olive oil) were without effect on rotenone inhibition. Cottonseed oil, which has vitamin E activity, had less effect than pure alpha tocopherol. It is proposed to test whether the anti-rotenone effect of alpha tocopherol is related to its vitamin E activity or to its anti-oxidant activity by testing other tocopherols. It appears significant at this point that colchicine and podophyllotoxin are antagonized by inositol, which, like vitamin E, has lipotropic properties. One possibility is that lipid metabolism supplies energy or metabolites essential to cell division in the sea-urchin egg. Another is that lipotropic agents provide an auxiliary energy source which the cell can use when normal pathways are blocked by mitotic poisons. The latter is supported by the antagonism of glucose for colchicine and podophyllotoxin. The pathways blocked by these poisons are not identical. Podophyllotoxin, which acts cytologically like colchicine and not like rotenone, is not antagonized by alpha tocopherol.

The movement of the cortical layer of Arbacia eggs in Ca-free medium through cleavage and the resting stage, as revealed by the shift of the pigment granules. KATSUMA DAN.

Previous studies on the movement of kaolin particles adhering to the outer surface of the Astriclypeus egg and also on the change in the cross-sectional width of the differentiated cortex of the Strongylocentrotus egg indicated that at the completion of the first cleavage, the cortical material accumulates around the base of the "stalk" between the two blastomeres. This material spreads and thins out along the surface during the resting stage, pushing most of the pre-existing cortical material toward the polar region. Immediately before the second cleavage, however, the thinned-out surface contracts again, pulling back the polar cortex and bringing about a more uniform distribution of the cortical material.

In *Arbacia punctulata*, many pigment granules are caught in the cortex at fertilization and serve as landmarks for the movement of the cortical layer. Soon after the first cleavage, the pigment granules accumulate densely around the stalk. Within 12 minutes (at 24° C.) the furrow region becomes practically colorless, the pigment granules all shifting in position toward the pole, where they pile up. Eight minutes later, or at 21 minutes after the beginning of the first cleavage, a tendency is perceived for the granules to move back toward the furrow. The granule density on the cell surface continues to become uniform until the second cleavage, which occurs at 24 minutes. Similar movement is repeated for the next cleavage cycle, although a complete redistribution becomes harder as the number of the blastomeres increases, *i.e.*, some pigment-poor area is left on the furrow side when the next division comes.

Cortical movement of this type is now well established, since the landmarks both outside and inside the cell show the same thing and, moreover, it is found in different forms of sea urchins.

Isotonic solutions for fish erythrocytes. JAMES W. GREEN AND JOSEPH F. HOFFMAN.

In a previously reported study on isotonic NaCl solutions for red cells of mackerel, tautog and smooth dogfish, it was shown that the densimeter technique of Parpart provided an accurate and rapid means for determination of isotonicity when compared with other methods. The method records the degree of swelling or shrinking of red cells in salt solutions of various

[1] Aided in part by an American Cancer Society institutional grant to the George Washington University Cancer Clinic.

tonicity. Using this technique the red cells of a number of the common Woods Hole fish were studied.

With teleosts the isotonic values depended upon the method of obtaining the sample of blood. With blood derived from the heart, the values for 14 different species ranged from 0.105 *M* for shark sucker to 0.262 *M* NaCl for mackerel, with the majority under 0.2 *M*. Blood from cut gills for 8 separate species ranged from 0.157 *M* for flounder to 0.274 *M* NaCl for tautog. The results with heart blood are thought to be closer to the true tonicity since the gill blood is probably contaminated with sea water.

For 5 different species of elasmobranchs isotonicity values ranged from 0.30 *M* for smooth dogfish to 0.38 *M* NaCl for the tiger shark. In all cases the blood was collected from either the heart or caudal vessels with no differences observed. In addition to NaCl some artificial solutions listed in the *MBL Formulae and Methods III* were studied. Knowlton's solution was found to be hypotonic; the "Elasmobranch" solution indicated an equivalence to 0.325 *M* NaCl; Van't Hoff's artificial sea water was equivalent to 0.38 *M* NaCl.

The lytic action of calcium ions on the pigment granules of the Arbacia egg.[1] PAUL R. GROSS.

Suspensions of Arbacia egg pigment granules can be prepared by homogenizing the eggs in the absence of free calcium. If calcium is then added to the homogenate, the granules break down, releasing a red pigment (echinochrome). This reaction is believed to be of importance for the life of the cell.

Since the lysis of the granules is accompanied by a color change, the reaction can be studied colorimetrically. By centrifuging the homogenates at high speeds (17,000 × g. for 10 min.) it is possible to obtain a clear supernatant fluid which contains the color. The absorption spectra have been studied and the most convenient wavelengths for the analysis determined.

The rate of granule lysis, but not the final percentage of granules lysed, depends upon the initial concentration of calcium. The reaction thus resembles hemolysis by surface-active agents. Relatively minute (10^{-3} *M*) amounts of calcium are required to initiate the reaction. Strontium and magnesium also induce lysis, but higher concentrations of Sr are needed, and still greater quantities of Mg ions. Ether, chloroform and soaps give the reaction in the absence of calcium.

When pigment granules are isolated, and washed 8–10 times in isotonic KCl, breakdown will no longer occur under the influence of Ca. Permitting the homogenates to stand for several hours at room temperature causes a sharp decline in the sensitivity to Ca. These observations agree broadly with those recently made upon the granules of *Paracentrotus lividus* by Hultin (1950). The latter worker's suggestion concerning the existence of a soluble, enzymic co-factor is further supported by the following: In the Arbacia material, the reaction is inhibited by Cu^{++}, Hg^{++}, cystine, and organic mercury compounds. Cyanide does not inhibit the reaction. There is a possibility that the co-factor is a protease.

Incorporation of deuterium into proteins of Arbacia eggs suspended in heavy water. HENRY D. HOBERMAN AND CHARLES B. METZ.

Fertilized and unfertilized Arbacia eggs were suspended in 10% deuterium oxide made isotonic with sea water. At designated times eggs were removed from the medium, preserved in alcohol, and later extracted with 5% trichloroacetic acid and with alcohol and ether. The water resulting from the combustion of this fat-free material was then decomposed to hydrogen. The deuterium content of this hydrogen was then determined by mass spectrometry. It was found that for the first 3.5 hours the rate of incorporation of deuterium into the proteins of fertilized eggs was only slightly greater than for unfertilized eggs. After this period the rate of incorporation of deuterium into the proteins of fertilized eggs approximately doubled. This acceleration was coincident with formation of the Arbacia blastula.

The maximum isotope concentration (C_m) which would be reached after complete turnover of the egg proteins was calculated. The value so obtained was equivalent to 10% of the deuterium concentration of the medium. Thus at least one out of every 10 stably bound hydrogen atoms

[1] This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, administered by Dr. L. V. Heilbrunn.

would be a deuterium atom at the time of complete turnover of the egg proteins. This suggests that in the blastula, proteins are synthesized from amino acids rather than from peptides. If the latter had been the case, the value for C_m would have been far lower than calculated. The data obtained in this experiment indicate that the half time of turnover for the proteins of the fertilized egg is approximately one day.

Some differential permeability effects of n-butyl alcohol. M. H. JACOBS.

It has previously been shown that n-butyl alcohol in sufficiently high concentrations induces in the erythrocyte a condition of cation permeability which may be regulated with considerable exactness by the concentration employed and the duration of the exposure. Under the conditions chosen for study the cells always undergo swelling followed by hemolysis.

More recently Ponder has reported that subhemolytic concentrations of butyl alcohol increase the permeability of the erythrocyte to potassium without causing it to swell. Though in several respects the two cases are not strictly comparable, it should be noted that swelling of a cation permeable cell might theoretically be opposed by a simultaneous reduction by the butyl alcohol of its normal anion permeability. That such an effect may in fact be present over a wide range of concentrations is shown by the enormous retardation by butyl alcohol of hemolysis in solutions of ammonium salts of strong acids whose entrance into the erythrocyte requires anionic exchanges, but not in those of weak acids where a molecular mechanism of penetration is involved.

A simultaneous appearance of hemolysis associated with cation permeability and protection presumably associated with reduced anion permeability can be demonstrated in erythrocytes treated with butyl alcohol in a solution of Na_2SO_4 to a point considerably short of hemolysis and then transferred to a solution of a sodium salt having a more rapidly penetrating anion, *e.g.*, NaCl. Erythrocytes so treated hemolyze rapidly, but the hemolysis may be checked by suitable sub-hemolytic concentrations of butyl alcohol. It is possible, though not yet proved, that the anti-hemolytic effect of certain other hemolytic agents may in part depend upon a similar principle.

Measurement of viscosity and elasticity of smooth muscles during contraction. WILLIAM H. JOHNSON AND JOHN F. PERKINS, JR.

"Viscosity" (mechanical resistance) and elasticity have been measured in the smooth muscles of Phascolosoma in a study aimed at clarifying the relation between the excitation and contractile systems in smooth muscles. An improved version of the apparatus previously described by us was used. The principle involves an analysis of the harmonic motion of one end of the muscle when a sinusoidal vertical motion is imparted to that end of the muscle through a weak spring. Our primary objective consists of a comparison of the viscous-elastic changes in a smooth muscle when activated by different stimuli including electrical, neurohumoral, ionic and cooling alone as a stimulus. Preliminary studies indicate that there are comparable increases in the viscosity and the elasticity of the pharyngeal retractor of the Phascolosoma with tetanic electrical stimulation on the one hand and with stimulation with excess potassium on the other hand. Our ultimate objective consists of measurement of the frequency and temperature dependence of viscosity and elasticity, in the hope of increasing our knowledge of the molecular changes occurring during contraction.

Rates of feeding and oxygen uptake in some filter feeding marine invertebrates. C. BARKER JØRGENSEN. See abstract in "Reports on Lalor Fellowship Research."

The internal hydrostatic pressure of the unfertilized Fundulus egg activated by puncture. CHIEN-YUAN KAO, ROBERT CHAMBERS AND EDWARD L. CHAMBERS.

These eggs, when stripped from the female and placed in sea water, spontaneously undergo activation until the blastodisc is formed. When punctured the activation is greatly accelerated. The unactivated eggs exhibit minute spherical vacuolar bodies, the platelets, lying on the surface

of the eggs underneath the chorion which is tough and non-elastic made evident by the fact that the chorion resists volume changes whatever the salt content may be of the environment up to that of sea water. Activation consists of the disappearance of the platelets concomitant with the appearance of a progressively widening perivitelline space between the chorion and the egg cell proper. The diameter of the egg cell decreases in proportion to the increase in width of the perivitelline space. The internal hydrostatic pressure of the eggs was determined by a mercury manometer connected through a column of water in a glass tube to a micropipette the tip of which, containing a short column of oil, was inserted into the egg under view through the microscope. The column of oil was maintained at the tip of the pipette by varying the pressure in the glass tube through the use of a 2 cc. Luer syringe connected at right angles to the glass tube. The pressure required to maintain the oil at the tip of the pipette was recorded by the height of the mercury in the manometer which, at the start, with the pipette tip in air, was 0 mm. Hg, *viz.*, one atmosphere.

The experiments consisted of inserting the tip of the pipette into an egg as soon as the egg was transferred from the mother into sea water. Within 3 minutes the pressure was found to be − 5 mm. Hg, indicating that the eggs were responding to being transferred to sea water which is hypertonic to the normal body fluid of the mother. As the platelets disappeared with the formation of the perivitelline space, the pressure progressively rose during the following 10–20 minutes until it reached a value of 150 mm. Hg which persisted during the following 60 minutes and longer. Positive pressures were obtained only in those eggs which had been activated, with the disappearance of the platelets and the formation of the perivitelline space. When the chorion is snipped open the fall of pressure indicates that the pressure is due only to conditions within the intact perivitelline space.

Metabolism of α-glyceryl ethers in marine forms. M. L. KARNOVSKY. See abstract in "Reports on Lalor Fellowship Research."

Vital staining by fluorescent dyes. R. KELLER.

Electric fields were investigated qualitatively by vital staining. The method used is built up on staining of test objects of uncontroversial electrotopography; the accumulation of dyes in biological objects of known fields gives a strong clue as to the distribution of electric charges in unknown fields. Conclusions drawn from studies of this kind have been supported by a long series of mineral analyses, determinations with electrometer and micromanipulator and with one-dimensional paper chromatography. Intracellular (basic) dyes are mostly travelling in aqueous solutions of high concentration in filter paper and in living protoplasm to the cathode, in dilutions of above 1 : 50,000 to the anode in both media. In diseases the whole intracellular (biologically positive) group diminishes in blood plasma and increases in organ parenchyma; the intracellular group was found to do the contrary. These changes may be taken as a characteristic consequence of the breakdown of electric potentials. Experiments with mice and chicken embryos were taken in partnership with S. S. Wanderman, who classifies fluorescent dyes in one-phasic and diphasic. The latter stain differently, dependent on their electric charge; for instance, thioflavin S is blue at strongly positive and yellow-green at strongly negative structures. Kodachromes were demonstrated of gall bladder, whose walls appeared strongly negative, of incubated chicken eggs with preponderantly negative embryos, of mouse cancers with negative growing neoplasms, with negative injury currents. The slides illustrate the possibility of applying dyes for the investigation of the otherwise invisible microscopic distribution of the electric charges of different tissues.

Iodine studies on Ascophyllum using radioactive iodine. SALLY KELLY AND NORMAN A. BAILY. See abstract in "Reports on Lalor Fellowship Research."

Distribution of P^{32} in Callinectes during the molting cycle. ELOISE KUNTZ.

The importance of phosphate in calcification processes during the molting cycle is well known but the distribution of phosphate for other metabolic changes which occur are less well

understood. The relative uptake of P^{32} by different tissues was measured two hours after injection at five different periods in the molting cycle: (1) immediately after molting, (2) 24 hours later, (3) during the inter-molt period, (4) at an early pre-molt (white line stage) and (5) immediately before molting (red line stage). Each tissue showed a different pattern of relative uptake of P^{32}.

The shell had a high content of P^{32} only at stage (2). Concentration occurred in the hypodermis in stages 2, 3, and 4, being greatest at stage 3. The gills concentrated P^{32} during stages 1, 3, and 4. The radioactivity of the hepatopancreas was low in stages 1 and 2, then increased during stages 3 and 4 with a very high content at stage 5.

The amount of P^{32} in the green gland varied widely between individual animals but was greatest during stage 5. The extremely high content of the green gland probably was the result of the re-absorption processes since no P^{32} was detected in the sea water in which the animals were kept.

There was a progressive increase of P^{32} in the heart from stage 1–5. Very little was found in striated muscle at any stage. The ventral nerve ring concentrated P^{32} at all stages with the greatest radioactivity occurring at stage 3.

The case against a correlation between flicker and metabolism of red cells. A. K. Parpart, J. F. Hoffman and J. W. Green.

Pulvertaft (1949) and Blowers, Clarkson and Maizels (1951) have observed that normal red cells when viewed by phase contrast microscopy show an active, continuous flicker-like alteration of optical density. They further claim that this "flicker" phenomenon disappears in the presence of metabolic inhibitors such as formalin (0.002%) and NaF (0.10 *M*). They concluded that there is a correlation between flicker and metabolism.

Normal mammalian and fish red cells examined by television microscopy in light between 3800 and 4500 Å show active flicker. Our observations lead us to believe, however, that the flicker is due to the active Brownian motion of the red cells. We have observed flicker in cells exposed to 1% formalin and 0.2 *M* NaF, both made up in isotonic NaCl. In most cases these agents cause the red cells to assume a more pronounced biconcave disc shape, and under these conditions the flicker is confined to the edge of the rounded portion of the cell. In fact, flicker is highly dependent on the shape of the red cell. Red cells in the form of flat discs (in Hayem's solution) show flicker at the outermost edge only. Increasing the viscosity of the suspension medium reduces the amount of flicker. Flicker is also present in cells that have been hemolyzed by a one-quarter dilution with water and then brought back to isotonic concentration. These and other observations force the conclusion that flicker is an optical phenomenon unrelated to the metabolic activities of the red cell.

The action of crystalline ribonuclease on phosphate labeled nucleic acid; a new assay method for ribonuclease. Jay S. Roth.

Phosphate labeled nucleic acid was prepared by growing yeast in the presence of P^{32} and isolating the nucleic acid by the method of DiCarlo. Several preparations having activities of 20,000–30,000 counts/min/mg. were used. The reaction rate was followed by the turbidimetric method of McCarty, and also simultaneous determination of the radioactive phosphate in the filtrate after removal of the precipitated acid-insoluble portion of the nucleic acid. Using a system containing 10 ml. of 0.1 *M* veronal buffer pH 7.6, 10 ml. of 0.2% labeled nucleic acid and 5 ml. of enzyme solution containing 1.25 γ of ribonuclease, it was found that the decrease in optical density of the solution and increase in radioactivity in the filtrate were both linear for the first 10 min. and then fell off rapidly. The relation between radioactive phosphate split off and enzyme concentration was also studied by incubation of 1 ml. of varying concentrations of enzyme with 1 ml. of buffer and 1 ml. of substrate for 30 min. at 25° C. This relationship was found to be linear for enzyme concentrations between 1×10^{-4} γ/ml. and 5×10^{-3} γ/ml. By using longer incubation times or substrates with higher specific activity it may be possible to increase the sensitivity considerably.

Ribonuclease may be conveniently assayed by this technique which has the following advantages: Increased specificity for the reaction, phosphate which may be split from cellular DNA

by DNA ases, or from phosphoprotein or phospholipid does not interfere. Turbid or colored solutions may be used and fewer controls are needed. No digestions or colorimetric determinations are required. Using this method, the presence of ribonuclease has been demonstrated in homogenates of Arbacia eggs, sperm and *Tetrahymena geleii* and also in intact cultures of Tetrahymena.

Changes in light emerging from the squid giant axone during activity: use of an intra-axonal light source.[1] SIDNEY SOLOMON AND JULIAN M. TOBIAS.

Toward the end of detecting, measuring and analyzing changes in protoplasmic ultrastructure occurring as a function of activity in nerve, light scattering techniques have begun to be employed. As a consequence, changes in light scattering have been observed to accompany impulse propagation. Partially to eliminate large sheath scattering and reflectivity parameters, a technique has been devised which minimizes these components and which should make the actual cellular changes more readily observable. A small quartz fiber, with white light impingent upon its protruding end, is introduced into the axoplasm of the squid giant axone, and, in this fashion, serves, at its impaling tip, to provide an intracellular light source. The light emerging from the axone at a right angle to its long axis has then been measured, using the optics of a standard microscope topped by a photomultiplier tube leading to a microammeter.

In 13 nerves, 85 periods of stimulation (175 per sec., 0.25–0.75 v., approx. 0.2 ms. pulses, duration 80–150 secs.) caused a decrease in emergent light in 61, no change in 23 and a small rise in 1. Precise time characteristics are not yet readily determinable. An initial increase in scattered light, as is often seen using whole nerve (see abstract by Bryant and Tobias, p. 205), has not been apparent in these experiments. The average maximum decrease in emergent light is about 3.3%, occurring after about 100 secs. of stimulation. Naively dividing by impulse number, this is about 0.0002% per impulse. Sub-threshold stimulation (17 trials) does not produce the change, nor is it seen with supra-maximal stimulation if the axone has failed to conduct impulses.

Light emergent directly over the needle tip has been observed, as well as the forward and back scattered light. The changes in these different sources seem to be not greatly different, one from the other, during activity. The data so far suggest that there may be a correlation between the amount of change and the initial axone diameter.

A physical study of the protoplasmic cortex of the Chaetopterus egg.[2] WALTER L. WILSON AND L. V. HEILBRUNN.

Many living cells consist of a mass of fluid protoplasm surrounded by a stiff layer just beneath the plasma membrane. This constitutes the cell cortex. In spite of the fact that the protoplasmic cortex is undoubtedly a most important part of the cell, little is known about its physical properties and how these may change during the life of the cell. The unfertilized egg of *Chaetopterus pergamentaceus* is excellent material for studying the state of gelation of the cortex. Imbedded in the cortex of this egg are numerous granules and these granules can be dislodged from the cortex by moderate centrifugal force. Following exposure to various agents, eggs are subjected to centrifugation and are then observed with high power magnification to determine whether or not the cortical granules have been dislodged. Eggs were treated with the following agents: potassium oxalate, potassium chloride, calcium chloride, acid sea water, sea water made basic with sodium hydroxide or ammonium hydroxide, ether, amyl alcohol, butyl alcohol, cold, heat, and ultraviolet radiation. Liquefaction of the cortex occurred following treatment with each of these agents except calcium chloride. Calcium produces a stiffening of the protoplasmic cortex. These results are similar to those obtained in earlier studies on the cortex of *Amoeba proteus*, thus indicating that the cortex of very different types of cells may be markedly similar in its physical properties.

[1] This work has been aided in part by a grant from the United States Public Health Service, Panel on Morphology and Genetics, and in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[2] This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, administered by Dr. L. V. Heilbrunn.

PAPERS READ BY TITLE

The role of the nucleolus in spindle formation. ROBERT DAY ALLEN.[1]

The germinal vesicle nuclei of the following marine eggs were studied cytologically: *Arbacia punctulata, Chaetopterus pergamentaceus, Spisula* (*Mactra*) *solidissima, Asterias forbesi* and *Nereis limbata.* Whole mounts of fixed material viewed with a phase contrast microscope revealed a double nucleolus (amphinucleolus of Wilson) as a common feature of all the eggs studied. This nucleolus consists in all cases of two parts: a bright nucleolus and one or more dark (usually smaller) nucleolini. The breakdown of the germinal vesicles was followed in all the above mentioned forms except Arbacia, where it occurs in the ovary. In all cases, the nucleolinus began to break up before rupture of the nuclear membrane and contributed to an increase in nuclear basophilia at that time. The nucleolinus or fragments thereof remained intact after rupture of the germinal vesicle and came to lie close beside the spindle area, which was surrounded by a field of basophilic granules from the germinal vesicle. Spindle fiber development is coincident with the final disappearance of the nucleolinus very close to the periphery of the spindle. Accounts of "nucleolar fragmentation" and of such fragments remaining in the spindle region are to be found in the older literature (Mead, Morgan, Tennant and Hogue, Scott, Lillie, Beams, etc.).

Indications of mucopolysaccharides have been reported in the mitotic spindle (Monné and Slautterbach) and in the nucleolus of Nereis (Kelly, using toluidine blue metachromasia). Accordingly, it was of interest to follow spindle formation with the reaction of Monné and Slautterbach. In Spisula eggs, the colors obtained were sufficiently strong to confirm the exchange of mucopolysaccharides between the disappearing nucleolinus and the forming spindle. The other eggs studied were too large to permit a clear staining reaction in whole mounts, although some eggs showed indication of mucopolysaccharide transfer. The results will later be tested on sectioned material.

Antimitotic substances secreted from eggs. ROBERT DAY ALLEN.[1]

Spisula (*Mactra*) *solidissima* eggs require several washings before they will fertilize and develop synchronously. Cleavage is delayed if thoroughly washed eggs are moderately crowded. Other marine eggs show this to a lesser degree (Glaser, Peebles, Lindahl). Spisula eggs constantly secrete a fertilization- and cleavage-inhibiting substance. Simple tests showed that this is not carbon dioxide or some other simple metabolic product. Since Spisula egg water inhibits fertilization and also agglutinates sperm, it evidently contains the "Mactra fertilizin" described by Metz. This substance is believed to be the active principle.

Fertilizin is evidently the egg jelly substance (Runnström, Tyler) which dissolves gradually in sea-water or rapidly in acid sea-water. As the clam produces large amounts of a very potent fertilizin on standing in normal sea-water, the preparations used here were supernatants from egg suspensions. Egg jelly contains a mucopolysaccharide and has the same anticoagulant properties as heparin; the same agents, including periodate, destroy these properties in both substances (Immers and Vasseur). In the present studies, Spisula egg water lost its anti-mitotic effect through treatment with 0.001 *N* periodate and subsequent dialysis. In order to test for mucopolysaccharides, the metachromatic reaction with toluidine blue was used; a strong positive correlation was found between metachromasia and anti-mitotic activity of egg water preparations (method of Wiame).

Spisula and Arbacia egg waters non-specifically inhibit the development of eggs of either species. These egg waters also have a similar but much less pronounced effect on Chaetopterus eggs, in which the secretion of a fertilizin has not been demonstrated. The present study suggests that the active anti-mitotic agent is identical with the sperm-agglutinating factor of egg water. However, in view of the non-specific character of the anti-mitotic effect, the two phenomena must depend upon different properties of this mucopolysaccharide. It is believed that the latter effect is due to its heparin-like composition.

[1] Fellow, National Cancer Institute.

Birefringence of granules in the epidermis of the leaves of Spinacia oleracea L.

RUTH P. ALSCHER AND GEORGE I. LAVIN.

Detailed morphological studies of the cells of the leaves of *Spinacia oleracea* L. were made using smears of ground fresh and frozen spinach leaves and preparations of epidermal tissue stripped from living leaves and washed with 0.51% isotonic sodium chloride solution. Frozen section preparations of fresh leaves and those fixed in 4% formalin were also observed.

It was found that the leaves have a cellular structure considered typical of a mesophytic dicotyledonous leaf.

However, it was observed that pigment granules are scattered at random throughout the cytoplasm of the cells of both the upper and lower epidermis. These granules are of two distinct types: light green (an average of 1.1 μ in diameter) and yellow green (an average of 1.2 μ in diameter). When viewed under polarized light, they are birefringent and light yellow in color.

Photomicrographs of surface and cross-section views of epidermal cells under polarized light indicate that the granules are composed of two distinct regions: a centrally located, birefringent region, which may be chlorophyll and lipid in nature; and a non-birefringent region, which may be protein in nature, located peripherally. This indicates that the granules are distinct morphological units and not mere droplets of pigment, and that they are morphologically different from starch grains and crystals. A polaroid polarizer and analyzer were employed. Kodak M plates were used; these were processed in D 19 developer.

These granules bear a striking resemblance, both in size, appearance, structure and birefringence, to those which Granick and Porter observed to be embedded in the protein matrix of chloroplasts of the mesophyll cells of spinach leaves. Therefore, these epidermal granules may be pro-chloroplasts, *i.e.*, the forerunners of chloroplasts, or they may be poorly developed chloroplasts.

Interaction of sodium and potassium ions with hemoglobin and with hemerythrin.

EDWIN H. BATTLEY AND IRVING M. KLOTZ.

The selective accumulation of sodium or potassium ions within erythrocytes has frequently been attributed to selective binding by the protein which constitutes the major component of the cytoplasm. Therefore the binding of alkali metal ions by crystalline hemoglobin and by crystalline hemerythrin was examined.

Crystalline horse hemoglobin (the authors are indebted to the Mass. State Antitoxin Laboratory for a gift of horse blood) was prepared by standard procedures except that the ghosts were flocculated and precipitated by lowering the pH to 5.0–5.5. After re-crystallization the protein was dissolved in distilled water and dialyzed 2 days against distilled water at 5° C. To measure alkali metal binding, solutions of hemoglobin containing 2–12% protein were equilibrated in a cellophane bag at pH 7.2 for 18 hours at room temperature against 0.001 *M* NaCl and 0.001 *M* KCl. Separate equilibrations were carried out also in the presence of 0.1 *M* NH_4Cl to overcome any Donnan effect. Sodium and potassium in both the hemoglobin solution and the dialysate were measured with the Beckman flame photometer. No binding was observed.

Crystalline hemerythrin was obtained from the coelomic fluid of *Phascolosoma gouldii* by alcohol fractionation of the hemolysate at 5° C. Aqueous solutions of hemerythrin, containing 0.663% protein and which were 0.01 *M* with respect to NaCl and to KCl, were centrifuged at 144,000 *g*. Binding should result in the presence of more sodium (or potassium) in the concentrated protein at the bottom of the centrifuge tube than in the supernatant. Analyses by flame photometry gave no evidence of binding. Analyses for sodium and potassium of whole, packed cells showed that Phascolosoma corpuscles concentrate potassium from the coelomic fluid. The values were 1.85×10^{-4} *M* K^+ and 3.41×10^{-4} *M* Na^+ per gram dry weight of cells, as compared to 0.38×10^{-4} and 3.8×10^{-4}, respectively, reported in the literature for body fluid.

Chromatographic analysis of fertilizin from the sand dollar, Echinarachnius parma.

DAVID W. BISHOP.

By means of the filter-paper partition chromatographic procedure amino acid and sugar assays have been made on sand dollar egg-water. This egg-water contains fertilizin capable of

reversibly agglutinating sand dollar sperm. In the material investigated the titer of this agglutinating substance in the fresh egg-water varied from 1/8 to 1/32. Essentially the same chromatograph pattern was obtained when eggs were allowed to stand in filtered sea water as when extracted with acid sea water, according to the usual procedures.

Amino acids were determined in dialyzed egg water, hydrolyzed 15–20 hours in 6 *N* HCl, and concentrated by partial evaporation. Two-dimensional ascending chromatograms were carried out on Whatman (no. 1) filter paper sheets. Solvents used were phenol-water (3:1 by weight) and n-butanol-glacial acetic acid-water (5:1:5 by volume). Ninhydrin was used as the developer. Ten spots have been provisionally identified by their R_F values as follows: isoleucine, leucine or norleucine, aspartic acid, and cystine; and probably methionine, tyrosine, threonine, glycine, arginine, and either lysine, serine, or glutamic acid. Unhydrolyzed material and sea water blanks gave no spots.

Sugar chromatograms were carried out on acid hydrolyzed egg-water, with n-butanol-glacial acetic acid-water (5:1:5) as the solvent, and ammoniacal silver nitrate as the developer. Each of four different hydrolyzed preparations gave a single spot, corresponding with the R_F value for levulose. In no case was fucose or galactose found, as reported by Vasseur for several European echinoderms. Unhydrolyzed preparations gave no spot.

This evidence suggests that fertilizin is a glycoprotein or a mucopolysaccharide (Tyler, 1948) and further contributes to the hypothesis of Vasseur (1949) that the specificity of fertilizin may lie in its polysaccharide constituents rather than in its amino acid content.

Certain of the hydrolysates, when neutralized and diluted with sea water, were capable of stimulating sluggish sperm into intense motility, probably attributable to the amino acids present.

The cytochrome system of starfish sperm. HANS BOREI AND CHARLES B. METZ.

On spectroscopic examination *Asterias forbesii* sperm suspensions present a perfectly normal cytochrome system. The positions of the absorption bands coincide with those of baker's yeast, but their relative strengths are different. Thus positions and relative strengths for the three cytochrome α-bands are ($Na_2S_2O_4$ added): ($a + a_3$) 605 (++), (b) 564 (+) and (c) 550 mμ (+++). This general pattern is similar to that found in sea-urchin sperms by Rothschild (1948), and reported in more detail by Keilin and Hartree (1949).

On addition of cyanide (in the presence of $Na_2S_2O_4$) the a-band is first sharpened up towards the red, indicating the formation of a non-absorbing $a_3 \cdots CN$-compound. The relative strengths of the bands under these conditions are: a (+++), b (++) and c (+++). When anaerobiosis develops, the absorption of $a_3 \cdots CN$ appears as a weak additional band around 590 mμ, or as a broadening of the a-band. If CO is bubbled through the suspension (in the presence of $Na_2S_2O_4$), a distinct band at 590 mμ can be observed, indicating an $a_3 \cdots CO$-compound. This band is easily bleached in the light beam of the spectroscope lamp (photoreversibility). Furthermore, the oxygen uptake of a sperm suspension (in an experiment of Sippel and Metz) was found to be inhibitable with cyanide. These observations establish the presence of the component a_3 (= cytochrome oxidase) and parallel those of Keilin and Hartree on heart muscle preparations.

A strong and broad pyridine hemochromogen band (++++) is obtained (after addition of pyridine + $Na_2S_2O_4$) around 555 mμ, and an additional weak band at 585 mμ.

The material was obtained from minced gonads of ripe animals, filtered through bolting cloth and diluted 1:3 with sea-water. An ordinary hand-spectroscope, illuminated with a tungsten ribbon filament lamp, was used. The light path was approximately 1 cm.

Further studies on growth and the processes of encystment and excystment in Dileptus. ANNA JOSEPHINE BRIDGMAN.

Animals of the genus *Dileptus* were excysted in the summer of 1950 and again in the summer of 1951 from soil collected in Waynesboro, Virginia. Tap or distilled water poured on the soil (which contains horse manure) will teem with these forms in a day or two.

It has been found possible to culture the animals in a balanced salt solution modified slightly from Loefer and brought to pH 8.0 by the addition of a phosphate buffer, when *Halteria* are added in some quantities as food. The *Halteria* are also grown in the balanced salt solution with added loops of *Aerobacter*.

The average size of the animals in cultures where they are multiplying is from 250–300 μ. The proboscis accounts for nearly half this length. There is a dorsal row of contractile vacuoles extending into the proboscis, and a spine at the end of the tapered trunk. The nucleus is long and sausage shaped. These characteristics are somewhat intermediate between those described by Kahl for *D. cygnus* and *D. gigas.*

A study of the encystment habits of these animals has so far revealed that when confronted with a diminishing food supply they may encyst or they may gradually decrease in size, perhaps occasionally dividing, until they are half or less of their original size. These small forms eventually encyst or die. This is in marked contrast to the situation in *D. binucleatus* where the tendency to encyst affects all the animals in a culture at approximately the same time, shortly after the depletion of the food supply. Perhaps as a corollary of this the cysts, some large and some small, show a much lower percentage of excystment with distilled water.

Oxidation-reduction dyes and "lithium larvae." MATILDA M. BROOKS.

In continuing the experiments on growth and development of marine eggs as affected by redox dyes of known electrode potentials, it was found that certain dyes initiated cleavage in unfertilized eggs of Arbacia, but that this process stopped at different stages and in most cases at the blastula stage. It was thought of interest to see wheher fertilized eggs would develop beyond this stage in the presence of these dyes.

It was found that two low potential dyes, indigo tetra sulphonate and indigo monosulphonate in concentrations from .01% to .0005% in sea water produced blastulae resembling the "lithium larvae" (extraverted gut and dumb-bell shapes). Most of them did not grow beyond this stage when kept in dye solution during two weeks of observation. A few developed abnormal plutei when transferred to sea water. The granules and gut were stained blue (indicating oxidized dye) and the cytoplasm was colorless (indicating either no accumulation or reduction).

Correlation of these results with reagents producing similar results as found by others is as follows:

Lithium reduces the rate of respiration of sea urchin eggs; CO and KCN stop oxidations; neutral red is a redox dye lower in the scale than the sulphonates; NaOH lowers the potential. One can conclude that the oxidative enzyme system is affected by all these reagents by lowering the redox potential which regulates metabolism. This permits the over-development of the micromeres at the vegetal pole of the egg, from which mesenchyme cells form. According to Child the vegetal pole has a lower metabolic gradient than the animal pole. Therefore these low potential dyes would stimulate the development at the vegetal pole and retard the development at the animal pole.

Several of these dyes at higher potentials, such as Guaiacol indophenol and methylene blue have not given such results in preliminary experiments.

A higher potential than that at the blastula stage is indicated for further development of the larva.

Hormones controlling the distal retinal pigment of Palaemonetes vulgaris. FRANK A. BROWN, JR., MILTON FINGERMAN AND MARGARET N. HINES.

Removal of one eyestalk from normal animals maintained on a white background was followed by a decrease in the degree of light adaptation of their distal retinal pigment to a significantly lower level within 15 minutes. Furthermore, dark-adapted one-eyed animals light-adapt at a significantly lower rate than normal and arrive at a final state of light adaptation comparable to that following removal of one eye as described above. These results clearly indicate that both eyestalks contribute to the normal control of the distal retinal pigment of both eyes. That the distal pigment is controlled, at least in part, by a light-adapting hormone is demonstrated by the fact that prawns dark-adapted and in darkness undergo extensive light adaptation in response to a one-minute exposure to bright light, the light adaptation becoming maximal in 30 to 60 minutes. A comparison of dark adaptation in one- and two-eyed prawns showed the rates and degrees to be indistinguishable. However, evidence for the presence of a dark-adapting hormone was obtained from two types of observations: 1) Widely different rates of dark-adaptation under different conditions, 2) dark-adapting influence of extracts of

eyestalks and central nervous organs. The extent of light adaptation and the rate of re-dark-adaptation in normal prawns in darkness following a one-minute light flash could be greatly increased by simultaneously injecting extracts of eyestalks of central nervous organs. This indicated that these organs contained sources of both a light-adapting and a dark-adapting principle. There is some evidence that the tritocerebral commissure contains the dark-adapting principle in virtual absence of the light-adapting one.

Failure to obtain "cytofertilizin" from Arbacia eggs. HELEN L. BYERS.

Most recent workers agree that the specific sperm agglutinating agent, fertilizin, is obtained only from the jelly surrounding Echinoid eggs. However, Motomura has recently presented evidence to show that the eggs of *Strongylocentrotus pulcherrimus* contain and can secrete another sperm agglutinating agent, "cytofertilizin," in addition to the extracellular fertilizin of the jelly. Since the relation of fertilizin to the egg architecture may have an important bearing upon the role of fertilizin in fertilization, Motomura's claim is of considerable interest. Therefore, an attempt was made to demonstrate the production of cytofertilizin by Arbacia eggs. Motomura's two principle experiments were repeated. Thus, samples of unfertilized Arbacia eggs were washed in acid sea water (pH 4.2) until the egg jellies were removed and the washes no longer agglutinated sperm. The eggs were then allowed to stand in sea water for 20 hours. The supernatants from such standing eggs (2 experiments) failed to agglutinate sperm. In a second series (3 experiments) unfertilized eggs were washed (acid sea water) free or nearly free of jellies, rinsed in sea water, fertilized and shaken to remove fertilization membranes (successful for 25–50% of the eggs). In one experiment the supernatant from the shaken eggs failed to agglutinate sperm. In two experiments the supernatants from the fertilized, shaken eggs contained sperm agglutinins but not in significantly higher titer than the supernatants from unfertilized control suspensions.

In view of these results it appears unlikely that ripe unfertilized or fertilized Arbacia eggs actively secrete a sperm agglutinating agent. Evidently fertilizin is obtainable only from the extracellular jelly of these eggs.

The mesoderm question in lobeless Ilyanassa eggs. ANTHONY C. CLEMENT.

In 1896 H. E. Crampton discovered that removal of the polar lobe from eggs of *Ilyanassa obsoleta* at the trefoil stage of the first cleavage results in the absence of a typical "mesoblast pole-cell," 4d, and later of the mesoblast bands. From this he was led to infer that the lobeless embryo consists only of ectoderm and endoderm. Subsequently E. B. Wilson ('04), in a study of the development of lobeless eggs of Dentalium, failed to find evidence of mesoderm in the partial larvae. It has been generally inferred, from the work cited, that removal of the polar lobe in these molluscs results in absence of mesoderm in later development.

I have removed the polar lobe at the trefoil stage in a large number of eggs of Ilyanassa and followed both the cleavage and later development. The characteristic mesentoblast cell 4d is absent, as Crampton reported. Despite this, the partial embryos and larvae arising from the lobeless eggs contain cells with the characteristics of mesenchyme, and they also develop an abundance of functional muscle tissue.

Two sources of mesoderm have been described in the classical studies of molluscan and annelidan cell lineage: 1) the primary mesoblast, from 4d; and 2) the larval mesoblast, from certain cells of the second and third quartets. In all probability the mesenchyme-like cells and muscle of the lobeless Ilyanassa larva are derived from the larval mesoblast.

Modification of the cleavage pattern in Ilyanassa through removal of the polar lobe. ANTHONY C. CLEMENT.

The highly complex and precise cleavage pattern of moluscs has long been recognized as a manifestation of precocious segregation in these mosaic eggs. In a careful comparison of the cleavage in normal Ilyanassa eggs with that in eggs from which the polar lobe had been removed at the first cleavage, I have found that the lobe is responsible for certain features of the cleavage pattern in at least three of the four quartets of micromeres.

In the normal egg the D quadrant micromeres are distinctive in several respects. For example, whereas the first quartet micromeres are similar in the A, B and C quadrants and their descendants show a similar pattern of subdivision, the D quadrant first micromere is smaller, it divides later and certain of its descendants divide at a slower pace and in a distinctive pattern. Peculiarities also distinguish the D quadrant micromeres of the second and fourth quartets.

Following removal of the polar lobe—a purely cytoplasmic structure—the D quadrant micromeres no longer possess the distinctive features mentioned; so far as they have been followed, they resemble the corresponding cells of the other quadrants and conform to the same cleavage pattern.

The type of cleavage modification impressed upon the D quadrant by the polar lobe varies from cell to cell. One cell may be made to divide earlier, another later and others remain unaffected. Similarly, one cell may be made larger, another smaller and others remain unchanged. Finally, the visible composition of the cell may or may not be altered.

At least two classes of cytoplasmic determinants may be postulated for the cleavage pattern in Ilyanassa: one, independent of the polar lobe and segregated according to the same pattern in all quadrants, is responsible for the differential cleavages within each quadrant; the other, stemming from the polar lobe, is responsible for the distinctive features superimposed upon the D quadrant.

On a dinitrocresol-resistant, phosphorylating mechanism present in cell-free homogenates of unfertilized Arbacia eggs. G. H. A. Clowes, A. K. Keltch and C. P. Walters.

Confirming and extending measurements by the techniques previously described the phosphate uptake which is coupled with oxygen consumption in cell-free homogenates of unfertilized Arbacia eggs with α-ketoglutarate or pyruvate as substrate is completely suppressed by 10^{-5} *M* dinitrocresol (DNC). With hexose diphosphate as substrate, 63 per cent of the phosphorylation is resistant to 10^{-4} *M* DNC and with pyruvate plus hexose diphosphate, 50 per cent (Table I).

Table I

Phosphorus and oxygen uptake are expressed in micromoles per 0.1 ml. original eggs in 60 minutes at 20° C.

Substrate	Conc. DNC	P uptake	O_2 uptake
	moles per l. $\times 10^5$		
α-ketoglutarate, 0.01 *M*	0	5.2	1.6
	10	0.2	1.5
Pyruvate, 0.01 *M*	0	2.6	1.1
	10	0	0.6
Hexose diphosphate, 0.0014 *M*	0	3.5	2.0
	10	2.2	2.2
	100	2.2	
Pyruvate, 0.01 *M* + hexose diphosphate, 0.0014 *M*	0	5.3	1.9
	10	2.9	1.7
	100	2.5	1.4

Homogenates of fertilized Arbacia eggs gave qualitatively similar results. Preliminary experiments suggest that homogenates of unfertilized Mactra eggs contain both a DNC-sensitive and a DNC-resistant phosphorylating mechanism. The authors thank Dr. M. E. Krahl for advice.

Phosphorus uptake of cell-free homogenates of unfertilized Arbacia eggs under aerobic and anaerobic conditions. A. K. KELTCH, M. P. SMYTHE AND G. H. A. CLOWES.

When homogenates of unfertilized Arbacia eggs are incubated with hexose diphosphate and pyruvate as substrate, the dinitrocresol (DNC)-resistant phosphorylation reported in the previous communication is apparently coupled with anaerobic disposal of hexose diphosphate, as the P uptake in a nitrogen atmosphere, with or without DNC, is approximately equal to that under aerobic conditions in 10^{-4} *M* DNC (Table I). This anaerobic phosphorylation is accompanied by very limited lactate production. The authors thank Dr. M. E. Krahl for advice.

TABLE I

P uptake, O_2 uptake, and lactate formation are expressed in micromoles per 0.1 ml. original eggs in 60 minutes at 20° C.

Conc. DNC	Aerobic			Anaerobic	
	P uptake	Lactate formed	O_2 uptake	P uptake	Lactate formed
Moles per l.					
0	7.2	−0.1	2.0	2.5	0.7
0.0001	2.9	0.1	1.3	2.2	1.0

On the effects exerted by calcium chloride and by freezing and thawing on the anaerobic, dinitrocresol-resistant, phosphorylating mechanism of homogenates of Arbacia eggs. G. H. A. CLOWES, A. K. KELTCH AND C. P. WALTERS.

The P uptake which is coupled with oxidation of α-ketoglutarate in homogenates of unfertilized Arbacia eggs is completely suppressed by $10^{-5} - 10^{-4}$ *M* dinitrocresol (DNC) and by 0.0007 − 0.006 *N* $CaCl_2$. The P uptake with pyruvate (0.01 *M*) plus hexose diphosphate (0.0014 *M*) as substrates is reduced only to the extent of 40–50 per cent by these agents (Table I).

TABLE I

P uptake, oxygen uptake, and lactate formation are expressed as micromoles per 0.1 ml. original eggs in 60 minutes at 20° C.

Preparation	Inhibitor	P uptake	Lactate formation	O_2 uptake
A. Homogenate from unfertilized Arbacia eggs	0	5.9	−0.1	2.5
	0.006 *N* $CaCl_2$	3.2	0.1	1.9
	0.001 *M* DNC	3.5	−0.1	1.8
B. Homogenate A frozen and thawed	0	3.3	−0.1	3.0
	0.006 *N* $CaCl_2$	2.9	−0.1	2.7
	0.001 *M* DNC	2.8	−0.1	2.5

After twice freezing and thawing of the homogenate, the P uptake falls from 5.9 to 3.3 micromoles and this residual P uptake is almost completely resistant to either DNC or $CaCl_2$ (Table I).

The anaerobic phosphorylating mechanism, which is resistant to DNC, to $CaCl_2$, or to freezing and thawing, appears to correspond to the water-soluble, phosphorylating mechanism obtained in the supernate after centrifuging cell-free homogenates of certain mammalian tissues at 20,000 g.; P uptake by this system is also resistant to the same agents and occurs anaerobically.

On the nature of the membranes in the egg of Saccoglossus kowalevskii: effects of certain chemical solutions. ARTHUR L. COLWIN AND LAURA HUNTER COLWIN.

The reactions of the three investing membranes of the fertilized egg of *Saccoglossus kowalevskii* to isosmotic concentrations of various salt-free and various salt-deficient solutions were studied. Repeated washings in the desired solutions were used to eliminate the original sea water environment.

The outer membrane (I) is unaffected by immersion in citrated calcium-free sea water but becomes hard to see and possibly undergoes actual swelling in a salt-free solution such as urea. The second membrane (II) remains intact in calcium-free sea water but, if treated during the period of membrane elevation, achieves its full expansion more rapidly than in the normal environment. Its reaction to urea is somewhat similar though much exaggerated. The third membrane (III), which is composed of large variously-shaped translucent granules in contrast to the transparent homogeneity of membranes I and II, disappears when immersed in any of the following solutions: calcium-free sea water, sodium chloride, potassium chloride, urea, sucrose. All other granules in the perivitelline space likewise disappear in these solutions. Upon return to sea water, membrane III does not reappear as an entity. However, numerous minute translucent granules do appear, and show Brownian movement. Some of these become aligned in the position formerly occupied by membrane III, some distance inward from the visible inner boundary of membrane II. The line is very delicate and does not resemble the original membrane III.

Changes of shape and position of the egg with reference to membranes following fertilization in Saccoglossus kowalevskii. LAURA HUNTER COLWIN AND ARTHUR L. COLWIN.

For about 10 minutes after their initial elevation during the fertilization reaction, the two outer membranes of the egg of *Saccoglossus kowalevskii* move away approximately equidistantly from all parts of the egg surface, simply expanding to a larger scale whatever irregular-oval shape the egg happens to have after having been shed. Then, as elevation continues, they gradually assume their "permanent" more or less spherical outline. The egg, too, becomes practically spherical during the post-fertilization period, and comes to lie near the center of the space enclosed by the membranes. Before this occurs, however, the egg undergoes a series of characteristic and orderly changes of shape and location (correlated chronologically with the separation of the first and second polar bodies). For a time it takes a very eccentric position, with the vegetal hemisphere lying very close to the membranes. In fact, there is an actual decrease in distance between the vegetal hemisphere and its surrounding membranes even though at this time the total area enclosed by the membranes is still increasing. The spherical shape and more nearly central position are finally attained before the first cleavage begins.

The occurrence of twinning in Fundulus heteroclitus after centrifugation. PAUL FORSTHOEFEL, S. J.

As part of the experimental work during the Teleost section of the Embryology Course at the Marine Biological Laboratory, Summer, 1951, several batches of *Fundulus heteroclitus* eggs were centrifuged at a force of 293 × g. (ca. 1550 RPM in an Adams Junior Safety-Head Centrifuge) for thirty minutes, at different times after fertilization. The eggs remained at the interface of layers of 0.73 *M* sucrose and sea-water during the centrifugation. 100 eggs centrifuged ten minutes after fertilization resulted in one case of twinning (1.0%), in which a main axis developed essentially normally, while a short supplementary axis at the level of the first somite showed head defects; both members of the pair had beating hearts. Forty eggs centrifuged 30 minutes after fertilization resulted in one case of twinning (2.5%), in which the members were joined at the tail end, and the axes formed a 180° angle; both twins were grossly normal in appearance. Three hundred-seventy eggs contrifuged at the early polar cap stage (ca. 30 mins. after fertilization) produced one case of twinning (0.27%), in which a small axis projected at a 90° angle at the first somite level of a larger but poorly differentiated axis; both embryos had small beating hearts in separate pericardial cavities. Of the 510 eggs centrifuged 10 or 30 minutes after fertilization, 3 cases of twinning (0.58%) resulted. Three

hundred eggs centrifuged at the 2-cell stage, and 420 eggs centrifuged when the embryonic axis was just visible, produced no cases of twinning. Numerous cases of abnormal and defective embryos were observed in the cultures of centrifuged eggs; no twinning or grossly abnormal embryos were observed in the control cultures. These preliminary results suggest that the period of formation of the blastoderm is particularly sensitive to the disorienting effects of centrifugation, and might indicate a splitting of an early "organization center" in the Fundulus egg.

The ingestion capacity of the wasp Habrobracon. DANIEL S. GROSCH.

A series of dissections in 10% formalin have elucidated feeding habits in the parasitic wasp *Habrobracon juglandis* (Ashmead) to provide a volume basis for dosage with chemical mutagens and radioactives. Starved animals will feed readily on certain dispensable syrups, especially honey. In such animals the abdomen is extremely flattened dorso-ventrally (see Figure 1, Grosch, *Biol. Bull.*, 99: 65–73). After a few minutes of feeding, the abdomen becomes full and rounded; however, there is a peculiar translucence to the part not apparent in a freshly eclosed wasp. An expansible crop which seems to function as a food reservoir is the structural feature involved. During feeding the crop is pumped full of material. At that time, as a relatively large ellipse, it occupies over two-thirds the abdominal volume. The wasp then loses interest in syrup until two days or later when wrinkles are apparent in the crop wall and its dimensions are slightly decreased. At this time fed material is demonstrable in the stomach. Before feeding, the crop is insignificant, wrinkled, flat, and asymmetrical (often approaching diamond shape). In samples of wasps of equivalent size, crop size varies within narrow definable limits (correlated with animal size).

A new method for determining calcium concentrations in biological materials. J. BRUCE GUYSELMAN.

The "Versenate Method," for determining total hardness in water, has been modified to give an accurate and extremely rapid technique for the measurement of calcium and magnesium in biological materials. With "Versene" (ethylene-diamine-tetra-acetic acid), and the appropriate indicator, it is possible to obtain either the combined calcium and magnesium content, or the calcium content alone.

The following method is a modification of the "Direct Versenate Calcium Titration," and has been adapted for micro-determinations of calcium in the fiddler crab (*Uca pugilator*). After obtaining the dry weight of an animal, it is ashed in a muffle furnace. The ash is then dissolved in 10 ml. of 40% HCl. A 0.1 ml. aliquot is transferred to a 125 ml. Erlenmeyer flask and diluted with distilled water, bringing the total volume to about 50 ml. To this is added 1.0 ml. of 4 *N* NaOH, 0.1 gm. of "CalVer" indicator, and then titrated with "Standard TitraVer" (prepared so that 1 ml. is equivalent to 1 mg. of calcium carbonate). The endpoint is a color change from orange to purple. It is advisable to run a blank, treating it in the same manner as the sample, since the reagents used are not entirely free of calcium.

The method outlined has less than 3% error for calcium concentrations ranging from 5 to 200 mg./10 ml. of acidified ash solution. (The error can be reduced by using larger aliquots.) The accuracy is quite adequate for the material being tested. Furthermore, the method is rapid; a single analysis can be completed in less than ten minutes.

Micropuncture and the localization of the subsequently-forming blastodisc in the unfertilized Fundulus egg. CHIEN-YUAN KAO.

The effect of puncture was tested on eggs stripped into: sea water, NaCl, KCl, $CaCl_2$, and a mixture of NaCl–KCl (18:2) isotonic with sea water. Activation occurred spontaneously, *viz.*, disappearance of platelets, formation of perivitelline space, and development of the blastodisc. At 60 minutes, a fully formed blastodisc appeared in practically all the eggs in sea water, and in only 50% in NaCl and in KCl. The blastodiscs in NaCl and in KCl, except for their granularity and brown color, were morphologically identical with those in sea water and in the NaCl–KCl mixture. In $CaCl_2$, the disappearance of platelets and formation of perivitelline space occurred much sooner than in sea water.

Using pipettes 5–15 micra in diameter at the tip, punctures were always made within a few minutes after stripping. In sea water and in the NaCl–KCl mixture the puncture had no effect on the localization of the blastodisc. In NaCl and in KCl, when the pipette was left continuously *in situ*, no typical blastodisc formed. However, there always occurred around the shaft of the pipette a broad-based zone of granular material which gradually thickened and became brown in color. This suggests that the puncture caused a protoplasmic movement peripherally toward the puncture. No other granular aggregation or blastodisc formed elsewhere. In some cases the pipette was removed immediately after making the puncture, the site of which could be identified by the presence of a small coagulum on the surface of the egg cell and extending through the punctured spot in the chorion. In these, a typically-shaped blastodisc always formed at the site of puncture. In $CaCl_2$ a granular aggregation occurred irrespective of the site of puncture. The surface of these egg cells within the chorion became excessively brittle, and ruptured spontaneously and easily, rendering it impossible for further observation.

Effects of x-ray and trypsin on the metachromasy of heparin-basic protein combinations.[1] JOHN W. KELLY.

Using a spectrophotometric method, the metachromasy (red color) of toluidine blue plus heparin can be studied quantitatively. The addition of basic proteins (protamine, histone) to a solution of toluidine blue and heparin destroys the metachromasy and produces a "potentially metachromatic" system. The effects of various agents on this system are of cytochemical and physiological interest.

Before trypsin is added to a solution of toluidine blue + heparin + protamine, the color of the solution is blue. Upon the addition of trypsin, there is a change of color from blue to red. This color change is related solely to the digestion of protamine by the enzyme; heparin is freed, and metachromasy is restored to the system. Trypsin does not affect heparin metachromasy. Both crystalline and crude trypsin behave in the same way, although crystalline trypsin acts in a decidedly lower concentration.

Both heparin and protamine are destroyed or changed by x-rays. In the case of heparin, this is indicated by lessened ability to produce metachromasy. In the case of protamine, the change is evidenced by a reduction in the heparin-neutralizing power of the protein. Stating this another way, irradiated protamine becomes less effective as an inhibitor of metachromasy. Approximate destruction rates of the heparin and protamine samples used were 1.7×10^{-4} mg./r and 2.2×10^{-4} mg./r respectively.

When heparin and protamine are irradiated together, the ensuing effect on metachromasy is apparently a resultant of (a) the individual destruction rates of the components, and (b) their concentrations relative to each other.

Ionizing radiation is known to increase the clotting time of blood, presumably by the release of a heparin-like anti-coagulant into the blood. If heparin is ordinarily combined with a more or less basic protein, the overall action of x-ray may under certain conditions be an effective release of this anti-coagulant into the bloodstream.

Metachromasy associated with various eggs and ovaries.[1] JOHN W. KELLY.

A survey was made of metachromasy in the eggs and in the ovarian tissue of 15 different species of animals. Toluidine blue was used as a stain both of living material and of sections of fixed tissue. Whenever possible, the eggs or oocytes and the ovaries were examined for the violet or reddish color characteristic of toluidine blue metachromasy. The biological material included *Amphitrite brunnea, Arbacia punctulata, Asterias forbesi, Arenicola marina, Chaetopterus pergamentaceus, Ciona intestinalis, Echinarachnius parma, Mytilus edulis, Nereis limbata, Opsanus tau, Palaemonetes vulgaris, Rana pipiens, Spisula solidissima, Uca pugnax*, and *Venus mercenaria*. Qualitatively, the observations varied greatly and are difficult to report briefly. Rarely the nucleus was metachromatic; more often the cytoplasm and especially the cortical region of the cytoplasm. The jelly surrounding eggs was commonly metachromatic and in all cases studied metachromasy was observed in some of the ovarian tissues.

[1] This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, administered by Dr. L. V. Heilbrunn.

The metachromasy observed is an indication of the presence of mucopolysaccharides (heparin, chondroitin-sulfuric acid, hyaluronic acid). Ovarian metachromasy in the forms studied is probably referable to a substance like hyaluronic acid, which is widely distributed in connective tissues. Metachromasy of the jelly, and possibly of the cortex, may be due to heparin-like substances. At any rate Immers and Vasseur have extracted from the jelly of a sea-urchin an anti-coagulant similar to heparin. Cytoplasmic and nuclear metachromasy cannot be so easily related to known cellular constituents.

The effect of forced aeration on the longevity of Laminaria. JOHN W. KING.

The difficulty of keeping Laminaria and other large marine algae alive in the laboratory is well known among phycologists. The writer felt that this difficulty could be overcome if the natural environment of the plants could be more satisfactorily duplicated in the laboratory.

Laminaria is usually found attached to rocks in areas where the water is in rapid motion. As the result of this rapid motion, large amounts of gases are collected from the atmosphere. With the natural environment of the plant in mind, a 1/16th inch hole was drilled in the nozzle of a salt water faucet. This hole was drilled in a downward direction at an angle of about 22.5 degrees. To the faucet was attached a rubber tube which extended to the bottom of a 4000 ml. beaker. The holdfasts of recently collected Laminaria were loosely but securely tied to rocks and placed in the beaker. The water was then allowed to run at full force throughout the experiment. Air was drawn in with a much greater force through the hole in nozzle than through a hole in rubber tube; therefore the hole in nozzle gave more desired results.

During the summer of 1950, *Laminaria agardhii* Kjellman was kept alive under the previously described conditions for a period of ten weeks with a minimum amount of sloughing off. A small amount of sloughing off could be expected since this normally takes place under natural environmental conditions as the plant grows older. At the end of ten weeks these plants were dried and they are now in the herbarium of the writer. This experiment was repeated for six weeks during the summer of 1951 and the results closely approximated those of 1950.

The skin as an organ of excretion in squids. BRUNO KISCH.

The experiments were performed on squids in the first days after hatching.

If small amounts of neutral red (about 0.01 to 0.005%) are added to the sea water, the little squids are intensely accumulating the color in their bodies and already a few minutes afterwards, colored droplets appear on the surface of the skin, usually first in the apical aboral part of the body. These droplets are growing and finally discharged. They fall down to the bottom of the container. The neutral red concentrated in these droplets is now fixed, probably combined with protein or mucous substances. It is heavier than sea water and no more soluble in sea water, as the added neutral red was.

This experiment can be repeated very easily for demonstration purposes, wherever newly hatched squids are available.

A cytological study of heat-activated Nereis eggs. ROBERTA LOVELACE.

Uninseminated eggs were activated by placing them in sea water at 35° C. for 1–5 minutes, or at 32–35° C. for 30–35 minutes. A cytological study of eggs fixed at various intervals after the termination of treatment shows that activated eggs receiving the shorter treatment (9 lots were studied) form both polar bodies, but neither nuclear nor normal cytoplasmic division occurs, and the chromosomes do not duplicate themselves. Visible components become segregated in these eggs; abnormal division into an oil-containing vegetal half and a granule-containing animal half sometimes takes place, and cilia develop on many of the eggs. Eggs receiving the longer treatment (16 lots were studied) may form both polar bodies, one polar body, or no polar body; the latter is of more frequent occurrence in all lots of eggs studied. Normal cleavages were achieved by some of the eggs in 50% of the treated lots, and a few normal trochophores developed in some of the lots with normally cleaving eggs. A cytological study of eggs from lots which gave rise to a few normal trochophores shows that eggs in normal

two- and four-cell stages formed both polar bodies and appear to be haploid. Eggs which formed no polar bodies did not undergo cytoplasmic segregation; they frequently divided abnormally and many developed cilia. These uncleaved "swimmers" contain a large mass of chromatin in the animal half of the cytoplasm. This suggests that chromosome duplication has occurred, but nuclear and cytoplasmic divisions have failed.

Some cytological effects of heat on Nereis eggs. ROBERTA LOVELACE.

Inseminated eggs of *Nereis limbata* were subjected to 35° C. for 10 minutes during two critical periods of early development: (1) During the first maturation division (25–30 minutes after insemination), and (2) during the first cleavage division (70–80 minutes after insemination). A study of eggs fixed at the beginning of treatment and at intervals thereafter indicates that 35° C. interrupts the first maturation metaphase in such a way as to produce stickiness of the tetrads and irregular scattering of the chromosomal clumps in the two asters. Subsequently these eggs may give rise to one large polar body containing a variable number of chromosomes, but frequently no polar body is formed. Lagging chromosomes are frequent at the first and second cleavage anaphases, and blastomeres with heteroploid chromosome numbers may result. Many abnormal multicellular larvae develop from these eggs, but some apparently normal trochophores are produced.

When treated during late prophase, metaphase or early anaphase of the first cleavage, nuclear division proceeds but cytokinesis is blocked and binucleate cells result. If the eggs are in late anaphase or telophase when treated, cleavage proceeds normally but is accelerated. In dividing binucleate cells, each astral center divides and primary spindles are established between daughter asters. Secondary spindles develop between adjacent asters and tetrapolar figures with four secondary spindles are frequent during anaphase. Cleavage of the binucleate cells is at right angles to the primary spindles, and the egg is divided into two equal binucleate cells. In successive cleavages, the two nuclei of a cell may fuse and tetrapolar figures with the tetraploid number of chromosomes arise. Most of the larvae developing from these eggs have abnormal pigmentation and abnormal oil distribution, but some normal trochophores were present in all cultures.

Glutathione in the body fluids of marine worms. SR. ELIZABETH SETON MACDONALD AND CHARLES G. WILBER.

An increase in reducing sugars in the body fluids of marine worms follows exposures to elevated ambient temperatures. It has been suggested that part of the increase may result from increased glutathione. Worms (*Phascolosoma gouldii* and *Amphitrite ornata*) were exposed to elevated temperatures in aerated sea water and the body fluids were then analyzed for amount of glutathione.

The mean values for the control worms in mg. reduced glutathione per 100 cc. body fluid are: *Phascolosoma*, 2.5; *Amphitrite*, 23.4. After exposure to elevated temperatures the mean values for *Phascolosoma* are in mg. per 100 cc.: 32° C. for 6 hours, 5.0; 35° C. for 1 hour, 5.9; 32° C. for 1 hour, 3.4, for ½ hour, 3.5. If the worms are kept at elevated temperatures for an hour and then returned to 22° C. for an hour of recovery, the elevated glutathione values persist.

Specimens of *Amphitrite* exposed to 32° C. for ½, 1 and 6 hours, respectively, give mean values in mg. per 100 cc. as follows: 20.2, 20.0, 20.9; if 1 hour recovery follows the 6 hour exposure, the mean value is 32.8. Exposures to 35° C. give the following results: ½ hour, 18.9; 1 hour, 27.6; 1 hour followed by 1 hour recovery, 24.7.

The results to date indicate that there is an increase in body fluid glutathione in *Phascolosoma* following exposure to high temperatures, but that no such increase is found in *Amphitrite*. In the latter there is even the suggestion of a decrease under these condtions.

There is a difference in the order of magnitude of glutathione values in the two forms: *Phascolosoma* controls are less than 5, *Amphitrite* are more than 20.

In the controls for *Phascolosoma*, there is a total of 12.7 mg. per 100 cc. of glutathione; of this, 10.2 mg. is oxidized. Reduced glutathione in this form consequently makes up about 19% of the total.

On the other hand, in *Amphitrite* controls there is a total of 27 mg. per 100 cc. of glutathione; of this, 5 mg. is in the oxidized form. Reduced glutathione in this form consequently makes up about 85% of the total.

It is evident that marked differences in glutathione exist between these two species. Whether the differences can be correlated phylogenetically or otherwise cannot be decided at this time.

Detergent action of sperm extract in Nereis limbata. W. J. V. OSTERHOUT.

The sperm of *Nereis limbata* (1) penetrates the tough vitelline membrane which is very resistant mechanically and does not dissolve in 5 *M* H_2SO_4 or in 8 *M* NaOH; (2) causes the extrusion of a large volume of jelly from the egg; and (3) makes the egg segment and produce swimming larvae (trochophores).

It has been found (Biol. Bull., 1950, **99**: 362) that duponol produces all of these effects. Hence it would be possible to account for the action of the sperm by assuming that it contains a detergent acting like duponol.

We can extract from the sperm a detergent acting like duponol as shown by its effect on surface tension. The sperm is shed dry and then covered with an equal volume of sea water and heated for a few minutes at about 50 to 75° C. A stream of air is then forced through the solution which produces a mass of bubbles resembling those obtained by treating a dilute solution of duponol in the same way.

The effect is apparently not due to the body liquid surrounding the sperm since it occurs only when the sperm is killed by heat or by exposure to distilled water.

When the freshly shed unfertilized eggs are placed in such sperm extract diluted to various degrees with sea water, the jelly is slowly formed in most cases.

Behavior of jelly in eggs of Nereis limbata. W. J. V. OSTERHOUT.

When unfertilized eggs of *Nereis limbata* were transferred from sea water to distilled water external jelly was formed (E. E. Just).

Such an extrusion of jelly could be completely suppressed when the unfertilized eggs were first transferred from sea water to various solutions at low pH values for a few minutes and then placed in distilled water. Chromic acid, hydrochloric acid, picric acid, osmic acid, mercuric chloride gave this effect. Ethyl alcohol inhibited the formation of jelly but in some cases a small amount was extruded.

When the unfertilized eggs were similarly exposed to potassium chromate solution at about pH 8, the formation of jelly in distilled water was not suppressed.

The external jelly formed in distilled water could be rapidly made to contract by the following solutions at low pH values: chromic acid, hydrochloric acid, tannic acid, mercuric chloride, potassium alum, and chrome alum. The contraction by dilute hydrochloric acid was tested for reversibility. If the contraction had not progressed too far it could be partially reversed by placing the eggs in distilled water. The jelly could be made to contract for a second time by again exposing the eggs to hydrochloric acid.

The external jelly formed in distilled water could be made to dissolve by placing the eggs in a solution of potassium chromate at about pH 8.

In view of these results it may be suggested that the jelly contains some protein.

The relation of the growth of oysters to different ecological conditions in Rand's Harbor. MADELENE E. PIERCE AND JOANNE E. CURRIER.

Rand's Harbor, Megansett, Massachusetts has a peculiar gradient in physical and chemical factors due to considerable fresh water dilution at the innermost reaches of the two basins. Large natural oysters occur abundantly at limited areas of the shoreline. In 1950, two-year old oysters were placed at four stations chosen to include the widest range of environmental conditions. One station was located in the fresh water inlet of the North Basin; one directly in the small, well protected North Basin in an area of fresh-water seepage; one in the larger, wind-swept South Basin, intermediate between the fresh water inlet and the outlet to the Bay; and the fourth at the Point where conditions more nearly approximated those of Buzzards Bay.

The growing period included a total of twelve weeks, extending from mid-July to early October. Measurements of the length, width, depth, and volume of 144 oysters were made at the beginning of the experiment, at the end of the first six weeks, and at the conclusion of the twelve-week period. Growth increases in length, width, and depth were fairly constant during the entire period. The rate of volumetric increases was significantly greater during the second six-week interval. Oyster growth was most rapid in the North Basin and at the Point. Negligible growth occurred in oysters placed directly in the fresh water inlet.

A study of the chemical, physical and biological factors present in the Harbor was maintained throughout the summer. Chemical conditions and temperatures were fairly uniform and within the favorable ranges for rapid growth rates. Physical factors, such as turbidity, molar activity and exposure, varied widely and may be considered the more important limiting factors of growth. Plankton studies indicated a plentiful supply of organic food material. Pests and competing forms were definitely found to be limiting factors in certain areas, particularly at the Point. The smaller North Basin was found most suitable to oyster growth. Although the substrate was exceedingly muddy, the area was sheltered from the direct influences of the wind and turbidity was low.

This study is being extended during the summer of 1951 to a total growing season of 18 weeks. The technique has been improved and more precise data are being recorded.

Sedimentation constant of hemerythrin. ROBERT A. RESNIK AND IRVING M. KLOTZ.

Crystalline hemerythrin was obtained by alcohol fractionation of coelomic fluid from the marine worm *Phascolosoma gouldii*. Sedimentation patterns of the protein dissolved in 0.1 *M* phosphate buffer at pH 7 were photographed in the Spinco ultracentrifuge operating at 1000 revolutions per second (corresponding to about 250,000 *g*). A single symmetrical peak was observed. The following sedimentation constants, S_{20}, corrected to 20° C., were computed.

Conc. of protein, %	S_{20}, seconds
0.100	6.01×10^{-13}
0.200	6.00×10^{-13}
0.50	5.64×10^{-13}
1.00	5.34×10^{-13}

Extrapolation of the sedimentation constants to infinite dilution gives 6.2×10^{-13} sec. If the molecular weight of this protein is 66,000 (Roche and Roche, 1935), the hemerythrin molecule must have a diffusion constant near 9×10^{-7} cm.^{2}/sec. and hence must be a very symmetrical molecule, even more symmetrical than hemoglobin.

The potency of rotenone and related compounds in inhibiting division of sea-urchin eggs.[1] EDWARD F. ROGERS AND IVOR CORNMAN.

Extract of the plant Ryania contains, in addition to the muscle poison and weakly anti-mitotic ryanodine, a much more potent inhibitor of cell division. Fractionation revealed this substance to be tephrosin, a compound closely related to rotenone in chemical structure and in insecticidal action. The concentrations of rotenoids which retard cleavage of Arbacia eggs are, in mg/L: rotenone 0.002–0.004; dihydrorotenone 0.002–0.2; deguelin 0.01–0.04; tephrosin 0.02–0.1; isorotenone 0.02–0.2; rotenolone I 0.02; rotenolone II 2.0. Dehydrorotenone and isorotenolone C were without effect at 1000 mg/L (in excess of saturation). These are close to the relative toxicities for fish and insects. Dihydrorotenone, which is active against cell division over a broader range of concentrations than the other rotenoids, is more toxic to fish than to insects. This points to a different mechanism of killing (or different criteria of death) in the two phyla. Synthetic insecticides were tested in connection with the proposal of Hummer and Kenaga that rotenone and DDT act via the same pathways. DDT and methoxychlor were without effect at 2 mg/L, while dianisyl neopentane retarded division at 2 mg/L. A thousand-fold difference in permeability must be invoked if these compounds act similarly within the dividing egg. The rotenoids are similar to flavones which act as gamones for Chlamydomonas.

[1] Aided in part by an American Cancer Society grant to the George Washington University Cancer Clinic.

Two were tested for ability to retard cleavage: isorhamnetin active at 0.2–2 mg/L and rutin, slightly inhibitory at 10–100 mg/L.

Cytochemical studies on mucous epithelial cells. R. R. Ronkin and E. Ronkin.[1]

Since mucous cells of the hypobranchial gland of *Busycon canaliculatum* (L), a marine gastropod, are abundant and large (average length, 200 μ), they were selected for studies on mucus formation and secretion. The commonest of several types of mucous cells in the gland is club shaped; its bulbous distal portion is almost completely filled with a mucous vacuole which extends basad into the long, slender, proximal portion of the cell, almost to the basement membrane. The mucus ends close to the elongated nucleus.

The extruded mucus and the vacuolar contents usually stained uniformly and metachromatically with toluidine blue 0, indicating the probable presence of organic, polysaccharide-linked sulfate. However, vacuoles in many otherwise similar cells were stained with the "normal" color of the dye, suggesting that intravacuolar mucus synthesis was not yet completed in these cells. Alcian blue 8GS, a non-metachromatic, "mucus-specific" stain, gave results which could be interpreted similarly. Vacuolar contents showed a marked loss of stainability (Bauer-Feulgen or toluidine blue 0) following digestion with hyaluronidase. There was no observable change after tryptic digestion. The extravacuolar cytoplasm contains yellow-pigmented granules and nadi-positive granules, both of various sizes. The distribution of the nadi-positive material suggests that cytochrome oxidase is more concentrated in the distal, more bulbous portion of the cell. Lipoid granules which were stained with Sudan black B are arranged in a broad band encircling the distal portion of the mucous vacuole. Although the connective tissue of the basement membrane was found to contain glycogen (Bauer-Feulgen; controls digested with saliva), we were unable to find any glycogen in the mucous cells. Furthermore, acid hydrolysis of mucous cells scraped from the basement membrane did not release reducing sugars in detectable amounts. These observations suggest that if glycogen(or galactogen) is not present, the final stages in the formation of mucus may be coupled with aerobic utilization of lipoid material in the distal portion of the cell.

The relationship between the state of distal retinal pigment and illumination in Palaemonetes. Muriel I. Sandeen and Frank A. Brown, Jr.

The distal retinal pigment of the prawn, *Palaemonetes vulgaris*, is highly responsive to changes in amount of illumination, migrating distally in darkness and proximally in bright light. Between some still undetermined lower limit of illumination, less than 0.001 ft.c. in animals maintained in white enameled pans, and approximately 1000 ft.c. under the same background conditions, the position of the distal pigment is a function of the illumination. The study of 285 animals distributed over a series of illuminations showed the pigment 94% towards complete light adaptation at 100 ft.c., 89% at 10 ft.c., 74% at 1 ft.c., 54% at 0.1 ft.c., 38% at 0.01 ft.c. and 26% at 0.001 ft.c. Under conditions of equal illumination of the containers in which the animals were kept, animals upon a black background show greater dark adaptation of the distal pigment than those upon white. This latter difference was clearly demonstrated to be not an albedo response but rather a direct response to the total illumination of the great preponderance of the retinal elements. The black containers, which reflected about 1/10 of the light reflected from the white ones, acted precisely like a reduction of source of light to about 1/10. Therefore, unlike for the integumentary dark chromatophores, general illumination of the eye and not albedo is the exclusive light factor influencing the state of the retinal pigments. The light source itself stimulates at any given instant a very small percentage of the total ommatidial complement, and appears to have correspondingly little influence in determining the general state of the distal pigment.

Respiration of Spisula eggs. Evelyn Sclufer.

The oxygen uptake before and after fertilization of *Spisula* (*Mactra*) *solidissima* eggs has been followed, using the Cartesian diver microrespiration technique. There seems to be no

[1]This work was supported by the Office of Naval Research under a contract with the University of Delaware.

rise or drop in oxygen consumption rate following fertilization. After fertilization the oxygen consumption gradually rises in an exponential manner. At the end of this period hatching occurs, at which time the rate is 4 to 6 times the pre-fertilization rate. Later on the rate curve flattens out. The oxygen consumption per egg and hour before fertilization is ca. 2×10^{-5} μl. The diameter of the egg is 55 μ. The material is very suitable for detailed studies of the respiration during early embryonic development; the egg is fertilizable after periods up to 6 hours measurement in the diver apparatus.

The effect of calcium and temperature on furrowing in Arbacia. ALLAN SCOTT AND RICHARD POLLEN.

If the width of the narrowing cell equator is plotted against time an S shaped curve results. Two procedures were used to obtain furrowing times: (1) precise photomicrographic measurements of the progressively changing rate; (2) visually estimating the time between the first indication of flattening and an equatorial diameter of 10 micra, closely approximating the straight line portion of the curve. Eggs were freed of their fertilization membranes. Calcium-free artificial sea water was prepared following Shapiro (1941) but adjusted to pH 8.2.

Furrowing is more rapid in Ca-free than in normal sea water. In sea water, furrowing times (method 2, 372 eggs) were 5.6, 10.2 and 20.1 minutes at 20.0°, 15.0° and 10.0° ± 0.5° C., respectively; in Ca-free sea water, furrowing (305 eggs) took 4.6, 8.1 and 14.6 minutes. Shapiro (1941, above) reported a 10% shortening of the 50% cleavage time in Ca-free sea water and implied that the developmental acceleration might be due to alterations in surface Ca-proteinates. We found a shortening of the furrowing time of 18% (20° C., method 2) in the absence of calcium but the times of 50% initiation of furrowing in Ca-free and normal sea water were within one half minute (6330 eggs). Thus we fail to confirm Shapiro in the degree of the effect.

The more rapid furrowing in Ca-free medium can be correlated with free cleavage in the absence of the hyaline layer. The simplest assumption is that the shortening of the furrowing time is primarily due to the removal of the mechanical restraint of the hyaline layer.

We have found no correlation between the rate of first cleavage furrowing in individual eggs and their rate of second cleavage furrowing, or between either of these and the time to their initiation of first cleavage.

Second cleavage furrowing averaged 3.5 minutes (Ca-free, 20° C., 97 eggs). Stratification does not alter furrowing times either in Ca-free or normal sea water. 10^{-4} *M* KCN, pH 8.2, which suppresses oxygen uptake by 50%, slowed furrowing times to 7.6 minutes (21 eggs, 20° C., normal sea water).

Preliminary measurements of birefringence changes due to electrical polarization of isolated squid giant axones. SAMUEL N. SHAW AND JULIAN M. TOBIAS.[1]

Excitation by the action current in nerve many have as one of its components a change in protoplasmic ultrastructure (molecular-macromolecular) which might be expected to entail a change in birefringence. To date, no such change has been found to accompany propagated impulses (Schmitt and Schmitt, Jour. Physiol., **98** (1940): 26). Since polarization by non-polarizable electrodes may mimic the electrical components of the propagated disturbance, however, and, because of increased intensity and duration, may make them more readily detectable (Tobias in *Modern Trends in Physiology and Biochemistry*, Academic Press, 1951), we have measured changes in birefringence of squid giant axones as a function of such polarization.

Axones were isolated in sea water and mounted in a mineral oil-filled, plastic, glass bottomed trough on sea-water-soaked thread electrodes. The polarizing voltage (15 v.) was applied via calomel half-cells, both current and voltage being measured throughout. Retardation was measured at 1–2.5 mm. from the electrode being studied by means of a Berek compensator mounted in a Spencer, polaroid, polarizing microscope. Diameter changes were measured with an ocular, filar micrometer.

[1] This work has been aided in part by a grant from the United States Public Health Service, Panel on Genetics and Morphology, and in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Normal axonal birefringence, using the whole sheathed axone, was found to be positive with respect to the axonal long axis, and was about 1.64×10^{-4} mu per mu. This compares favorably with figures given by Schmitt, Bear and Young, 1.5×10^{-4}. Initial cathodal polarization produced a fall in birefringence (1.9×10^{-6} mu per mu per min., aver. of 5); initial anodal polarization produced a larger fall (4.6×10^{-6} mu per mu per min., aver. of 4). Cathodal polarization, if it followed anodal polarization, however, in all cases favored a rise in birefringence, whereas anodal polarization following cathodal polarization again produced a fall. Spontaneous changes following cessation of the polarizing current have not yet been measured, and these are required before much significance may be attributed to these apparent reversals.

Reduction of neotetrazolium chloride by X-radiated root tip cells of Allium. B. P. SONNENBLICK.

Reports by several investigators (Antopol, Glaubach, Goldman, Narahara, Sonnenblick) indicate that the enzyme systems responsible for the reduction of neotetrazolium chloride (NT) are present in many living tissues. The faintly yellow salt undergoes a progressive series of color changes and is finally converted to an insoluble purple-black granular compound located characteristically in the cytoplasm about the nuclear membrane.

Studies on the mechanism of NT reduction show that active —SH groups play a significant role in the process. Since reports exist indicating that moderate amounts of X-rays effect inhibition of sulfhydryl enzymes *in vivo* and *in vitro*, it was decided to study the reduction of NT by X-radiated root tip cells. Dormant bulbs and actively growing roots were irradiated and excised roots subsequently tested at various periods following exposure. Roots were given 300, 600 or 900 roentgens, while bulbs were exposed to 300, 1200 or 2400 roentgens.

Tests indicate that cells given treatments causing a moderate frequency of chromosomal aberrations show no inhibition in the speed and intensity of NT reduction. With dosages causing severe nuclear damage the results are variable, but even after such exposures NT reduction can proceed as well as in control material. To interpret these results the following must be considered: (1) the greater sensitivity of dilute aqueous solutions of enzymes to irradiation than when accorded the protection of other substances within cells, (2) the possibility of unequal inhibition of different sulfhydryl enzymes by X-rays, and (3) the role played by other than —SH enzymes in NT reduction.

Very recently Le May of Cambridge University reported no inhibition of the —SH enzyme succinic dehydrogenase in rat kidney slices and homogenates following 400 r or 800 r of total-body X-radiation, a result *in vivo* contrasting markedly to the effect *in vitro* of X-rays on dilute solutions of that enzyme.

A blood anti-coagulant from surf clams.[1] LYELL J. THOMAS, JR.

According to Heilbrunn many of the colloidal changes taking place in cells are similar to those which occur in the blood clotting process. If this is true, then one would expect to find both clotting and anti-clotting substances in all types of living systems. For this reason I have been examining various invertebrate materials for their content of heparin-like substances. A potent blood anti-coagulant has been obtained from the common surf clam, Spisula. This anti-coagulant gives a strong metachromatic reaction with toluidine blue which is an indication that it is chemically similar to heparin.

After removal of shell, visceral mass and gonads, the clams were extracted by the method of Charles and Scott for the isolation of crude heparin. This was followed by a phenol-water partition as recommended by Homan and Lens. The product obtained in a yield of about 500 mg. per kilogram still contains colored impurities and a trace of protein material.

Anti-coagulant activity was assayed by a method similar to that of Chargaff. Untreated hen plasma diluted with test solution and thromboplastin formed the clotting system. All preparations from Spisula were the equivalent of or better than ordinary commercial preparations of heparin (Hynson, Wescott and Dunning).

[1] This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, administered by Dr. L. V. Heilbrunn.

The metachromatic activity of the Spisula preparations and of various commercial heparins was tested for me by Mr. John W. Kelly. In all cases, metachromatic activity paralleled anti-coagulant activity.

Further work is under way to determine the chemical nature of this substance from Spisula and to determine whether it can be obtained from other invertebrates.

Phosphorus metabolism in developing sea urchin embryos as a function of temperature. CLAUDE A. VILLEE AND DOROTHY T. BALZER.

Previous experiments had shown that low temperature markedly reduced the uptake of P^{32} by fertilized *Arbacia* eggs and it was concluded that phosphorus enters the cell by an enzyme-controlled metabolic process rather than by simple diffusion. In the present experiments, eggs were fertilized in sea water containing 3 to 5 μC of P^{32} and incubated seven hours at controlled temperatures ranging from $-12°$ C. to 28° C. After incubation they were centrifuged lightly to remove the sea water, homogenized in ice-cold 7% trichloracetic acid and fractionated by a modified Schmidt-Thannhauser method. The specific activities of the fractions were calculated from Fiske-SubbaRow phosphorus determinations and P^{32} analyses made with a dip counter. When the logarithm of the specific activity of the RNA P is plotted against the temperature, a straight line is obtained. Thus, the incorporation of P^{32} into RNA is described by the equation, Specific Activity $= e^{kt}$, with k having a value of 0.036. Similar plots of the other fractions did not give straight lines; the plot of the acid soluble P fraction fell below a straight line at higher temperatures and the plots of the DNA P and phospholipid P increased with temperature more than by a straight line relationship.

Studies of phosphorus metabolism in ovarian eggs of the salamander, Triturus pyrrhogaster. CLAUDE A. VILLEE AND WILLIAM R. DURYEE.[1]

Ovarian eggs were dissected free from ovarian tissue in amphibian-Ringer's solution and separated into two groups (small, 1.2 mm.^{3}; large, 6.3 mm.^{3}). About 200 eggs were placed in 35 ml. of bicarbonate-buffered Ringer's (pH 7.6) containing 1 μc/ml. of P^{32}. After 6 or 24 hours, the eggs were removed, rinsed in Ringer's, blotted and weighed. They were homogenized in ice-cold 7% trichloracetic acid and fractionated by a modified Schmidt-Thannhauser procedure. Phosphorus determinations were made by the Fiske-SubbaRow method and P^{32} determinations with a Solomon-Estes dip counter.

Salamander eggs differ from Arbacia eggs in having a large phosphoprotein fraction; 74% in small and 90% in large eggs of the acid insoluble phosphorus is phosphoprotein phosphorus. In both sizes the RNA phosphorus turns over faster than the DNA phosphorus. In small eggs (without yolk) the phosphoprotein phosphorus turns over faster than either RNA or DNA, but in large eggs (with yolk) the phosphoprotein phosphorus turns over at a slower rate than either RNA or DNA. Both large and small eggs show similar rates of turnover in their acid soluble P fractions and in their phospholipid P fractions.

Large eggs have considerably more phospholipid P than small ones, whether calculated as mg. P per gm. wet weight of eggs or as μg P per egg. Large eggs have smaller amounts of DNA P and RNA P than small ones, calculated as mg. P per gm. eggs: namely 2.65 times as much DNA P and 2.05 times as much RNA P. Both DNA and RNA P turn over more slowly in the large eggs than in the small ones.

Feulgen stains show that the nucleus is nearly DNA-free, except for the vesicle cores of each nucleolus. Subsequently, nucleolar DNA is extruded through the nuclear membrane and disperses in vacuoles toward the cell surface.

Diurnal rhythm in the controlling mechanism of distal retinal pigment of Palaemonetes. H. MARGUERITE WEBB AND FRANK A. BROWN, JR.

Palaemonetes maintained in constant darkness exhibit a diurnal rhythm in the characteristics of their response to a one-minute flash of 250 ft.c. illumination. This takes the form of variations in the extent of light adaptation and in the rate at which the animals return to a condition of

[1] National Cancer Institute, U. S. Public Health Service, Federal Security Agency.

complete dark adaptation. This rate is maximal at approximately midnight, rapidly decreases to a minimum at 5 or 6 A.M., increases at first rapidly and then more gradually until 7 or 8 P.M. when there is a second rapid increase to the maximum rate at midnight. It is reasonable to assume that the variations in the extent of light adaptation and the rate of subsequent re-dark-adaptation following one-minute light flashes were due to differences in the availability of dark-adapting hormone in the 24-hour cycle since animals maintained in constant darkness show approximately equal ability to light-adapt throughout the 24-hour cycle. Animals maintained under conditions of continuous illumination of 250 ft.c. are able to dark-adapt at nearly equal rates at any time during the 24-hour cycle, indicating a ready availability of the dark-adapting hormone. It is concluded that constant illumination at 250 ft.c. suppresses the rhythm of availability of dark-adapting hormone. A persistent rhythm is directly observable in normal Palaemonetes under conditions of intermediate states of light adaptation such as obtains at 10 ft.c. on a black background. Under these latter conditions this rhythm was of significant but small amplitude involving about 25% of the normal total pigmentary range.

The effects of x-rays upon dividing and non-dividing cells: Paramecium caudatum.

RALPH WICHTERMAN.[1]

Clonal cultures of *Paramecium caudatum* were cultivated in desiccated lettuce infusion and inoculated with *Aerobacter aerogenes* as the food source. While division may be completed one-half hour from the time a spindle-shaped dividing cell can be first recognized, the process may last 2 or more hours before final separation into two daughters. In the very late division stage in which the cell is almost completely constricted into two, the forming daughters may be held together by a fine strand of protoplasm for an hour or more before breaking. In contrast to vegetative cells, the dividing cells from heavily bacterized cultures are sticky and readily adhere to the walls of capillary pipettes. In such rich cultures, chains of 3 or 4 cells are commonly seen. Like vegetative specimens, such chains of fully formed but occasionally smaller animals swim in left spirals. Like typically dividing paramecia, the chains are more abundant at the bottom of a culture.

Many isolated late dividing and non-dividing (vegetative) stages were irradiated with 300,000 r, immediately examined and maintained in isolation culture at 24° C. Irradiation of late dividing cells greatly prolongs the division process. Such dividers commonly remain suspended in division, yet swim actively, for 6–48 hours, occasionally longer. Generally the X-rayed dividing cells do not live to produce daughters but if produced, they persist for a day or more then die. Occasionally one of the daughters produced by an irradiated divider dies while the remaining daughter lives to divide later into two. This may be repeated but specimens usually die. In exceptional instances, an irradiated divider after gradual recovery from X-ray effects may produce viable daughter cells which yield survivors that later approach the control fission-rate. Irradiation of dividers can produce giant C-shaped monsters from which normal appearing specimens may originate. While irradiated dividing cells commonly die during the process of division, when given the same X-ray dosage non-dividing cells from young cultures commonly live to produce viable progeny after overcoming an initial division lag. However, non-dividing cells from older cultures are almost instantly killed by the same X-ray dosage. Irradiated, bacterized culture fluid has no lethal effect upon paramecia.

The effects of x-rays upon conjugant and vegetative stages of Paramecium calkinsi.

RALPH WICHTERMAN.[1]

Clones of brackish-water *Paramecium calkinsi* of opposite mating type have been maintained in continuous cultivation over 4 years in lettuce infusion-sea water cultures inoculated with *Aerobacter aerogenes*. When in proper nutritive condition, specimens of opposite type, when mixed, form the characteristic clumps, enter into conjugation any time of day and at 25° C. separate 12–13 hours later. Lower temperatures lengthen the process.

Irradiation of joined conjugants applied at the rate of 7420 r per minute until 250,000 r are reached prolongs the conjugation process. Most of the irradiated conjugants recover but do

[1] Aided by a grant from the Committee on Research and Publication, Temple University and performed under Contract NR 135–120, Office of Naval Research, Department of the Navy.

not begin separation into exconjugants until all of the control conjugants have separated. In a sample experiment using 108 isolated control conjugants, all separated into exconjugants within a period of 2 hours completing the process within 13 hours. On the other hand, 112 irradiated conjugants separated over a 5 hour period with the last conjugants separating approximately 22 hours after mixing the opposite types. Unirradiated vegetative and conjugant stages characteristically spiral to the right in this species. The same is true for the irradiated conjugants even in late stages where a thick strand of protoplasm is all that holds the conjugants together in the region of the paroral cones.

Recent, early stage conjugants, conjugants near the completion of the process and young exconjugants were irradiated to determine possible differences in susceptibility to the X-rays. No well-defined difference could be detected. Most of the irradiated young and old conjugants and the young exconjugants survive. However, with vegetative stages from a culture of one mating type reactive for mating, fully 90% of the irradiated specimens die after 24 hours. The survivors overcome the initial division lag—not dividing for 3 days—then the fission-rate gradually approximates that of the controls. When specimens are taken from mating-reactive cultures and irradiated, there is seen to be a difference in X-ray susceptibility in the opposite mating types. With one mating type, nearly all the vegetative stages are killed almost immediately after maximal dosages while comparatively few are killed from those of the opposite type even when both are of the same cultural age. Irradiated, bacterized culture fluid has no lethal effect upon the paramecia.

pH determination in the giant nerve fiber of the squid. FLOYD J. WIERCINSKI.

The problem of determining the exact pH of nerve tissue presents numerous difficulties. Most important of all, most nerve tissues are opaque to light so that it is impossible to use pH indicators. The giant nerve fiber of the squid offers some possibility for obtaining some data for the determination of the pH of nerve axoplasm with indicators since the fresh tissue is transparent to light.

A squid was decapitated. The mantle tissue was immersed under mineral oil; pH determinations were attempted *in situ* by injecting indicators into the nerve with a quartz micropipette. In later experiments the giant nerve was dissected out from the mantle under oil. The sheath was removed from sections of the nerve. The nerve was then prepared in a hanging oil drop for microinjection. The water soluble indicators were used. Reference was made to micro-color standards for the pH reading. The results were recorded on Kodachrome film.

Tests with phenol red showed a pH < 7.0, brom thymol blue > 6.4–6.8, brom cresol purple > 6.0–6.8, brom cresol green > 5.6, chlor phenol red > 6.0, brom phenol blue > 4.6.

The results obtained indicate a pH somewhere in the neighborhood of neutrality for the clear axoplasm of the giant nerve fiber of the squid that is put under oil and measured immediately. The nerve sheath and stellate ganglion indicated a more alkaline pH reading. Crushing a nerve fiber not under oil showed more acid pH readings.

Activity of chromatophores in the integument of the squid. FLOYD J. WIERCINSKI.

A squid was cut across the head immediately posterior to the eyes. This cut separates the cerebral ganglion from the pleuro-visceral ganglion. It was noted that the chromatophores expanded to a dark red color. The decerebrized squid carried on the function of locomotion for a short period of time. Ink invariably was released. Later the chromatophores contracted and the squid appeared white in color.

A squid was decapitated by a cut made across the neck posterior to the pleuro-visceral ganglion. The chromatophores immediately contracted and the squid became white. In most cases ink was not released and the squid lost all locomotion.

Pieces of integument were removed from a squid. When viewed under the compound microscope the chromatophores appeared to be inactive but in a few minutes they began to contract and expand. This phenomenon seemed comparable to tissue shock.

Some chromatophores appeared quiescent and contracted, others were active at a rate of about 8 to 12 contractions per minute. They appeared in various shades of yellow, red and black. Radiating strands of muscle fibers attached to each chromatophore were seen. When the chromatophore was expanded the muscle fibers were in contraction. These phenomena were recorded on Kodachrome film.

A number of substances were tried on the activity of chromatophores in the excised epidermis of the squid. 0.1 *N* HCl fixed the chromatophores in the contracted state and the muscle fibers in the extended state. *M*/2 NH_4Cl in sea water, pH 6.55 changed the rate of contraction of a dark red chromatophore from 8 per minute to 42 per minute. A 1:100 heparin in sea water increased the frequency of contraction; 1:500 hyaluronidase had some effect in causing expansion of the chromatophores (muscle fibers contracted).

It is concluded that a chromatophore in the integument of the squid is contracted and expanded by means of muscle fibers. The cerebral and pleuro-visceral ganglion play a role in keeping the muscle fibers contracted. The chromatophores are then in the expanded state. A new approach to the study of muscle contraction may be found here.

Lipids in frog blood. CHARLES G. WILBER, JOEL R. LIEB AND G. M. SLANE.

In connection with a survey of the lipid constituents of body fluids from a wide variety of animals, a study of the lipids in the blood of various frogs was begun. Routine colorimetric procedures were done. In the species *Xenopus laevis* the mean blood fatty acids are 520 mg. per 100 cc. whole blood, with a range of 490 to 540 mg. per 100 cc. Lipid phosphorus in this form averages 20.7 mg. per 100 cc. with a range of 16 to 27 mg. per 100 cc.

In *Rana pipiens*, erratic results for total fatty acids of the blood have to date prevented any reporting of these values. Lipid phosphorus of the blood from frogs kept at 18° C. for several weeks averages 10.5 mg. per 100 cc. with a range of 9 to 12 mg. per 100 cc. In frogs kept at 27° C. for 24 hours the average blood lipid phosphorus is 8.9 mg. per 100 cc. with a range of 7.5 to 10.5 mg. per 100 cc. In frogs kept at 35° C. for 24 hours the average blood lipid phosphorus is 11.2 mg. per 100 cc. with a range of 9 to 13 mg. per 100 cc. Although the mean values suggest a change of lipid phosphorus content of frog blood with increased temperatures, the differences are not statistically real. These results are not in accord with those on the turtle in which the blood lipid phosphorus decreases with increased ambient temperatures. It is, moreover, apparent that the average value of lipid phosphorus in the blood of Xenopus is closer to that of the turtle (19 mg. per 100 cc.) than of the frog Rana. The significance of this latter fact is not clear.

Protein molecules with relatively low molecular weights? DOROTHY WRINCH.[1]

Despite the vast variety of sizes and shapes of native protein structures, evidence compatible with the idea of one or a homologous series of molecular skeletons characterizing proteins in general is accumulating. The variety would then be interpretable in terms of *particle* structures comprising a wide range of different numbers of the common skeleton or skeletons, variously substituted and variously arranged.

The insulin structure, MW ca. 36,000, is known to be a trimer of structures with estimated molecular weight 11,700 (Low, 1949): but subsequent work (Fredericq and Neurath, 1950) suggests there is a further dissociation, into entities with MW ca. 6000. Such an entity would have a *skeletal* residue number of about 48–50, following Chibnall's amino acid analysis and allowing for the *extra-skeletal* residues with free α-amino or α-carboxyl groups. This result accords well with the previously made suggestion of the possibility of 6 cages each comprising 48 skeletal residues plus extra-skeletal residues in the original insulin structure (Wrinch, 1948).

Such a type of low molecular weight entity throws light on the case of horse myoglobin, a structure with ca. 147 residues. It seems impossible to interpret the vector maps derived from this X-ray study, except on the assumption that the structure is a particle with more than 2 sub-units. A structure comprising 3 sub-units would allocate about 49 residues to each, fitting well with the proposed entity. Other proteins which may well fit into the picture of this single type of entity, in various numbers and in various arrangements, include whale myoglobin, horse hemoglobin, pepsin and β-lactoglobulin and even such relatively large proteins as edestin, tobacco seed globulin and excelsin.

Further confirmation of this viewpoint is being sought in a detailed study of the intensity maps obtained in X-ray studies of wet crystalline proteins.

[1] This work is supported by the Office of Naval Research under contract N8onr–579 with Smith College.

Water in protein crystals. DOROTHY WRINCH.[1]

It is well known, from X-ray studies, that crystalline proteins containing prodigious complements of water yield intensities which indicate a high degree of orderliness extending down to atomic dimensions. For wet insulin, there is no less than 46 per cent water by weight, if a dry molecular weight of ca. 36,000 be assumed. For horse methemoglobin, still larger complements of water are present and crystals yielding intensities down to atomic spacings. The effect on the intensity maps of reducing the water complements shows that not only the protein structures but also some considerable proportions of the water molecules form orderly patterns at the atomic level. It follows that the role played by the water molecules in determining the intensities, and the vector maps derived from them, is likely to be of crucial importance.

Accordingly a study of the vector maps to be expected of molecular crystals containing a patterned medium has been undertaken. It proves possible to trace the accentuation of the nodes of the lattice of the medium and to determine the manner in which it depends upon the particular spaces occupied by the molecules themselves. In an application to the case of wet insulin, it is found that the well-marked peaks in the vector map at about 5A from the origin may be due in part to the existence of water molecules in clusters within the crystal.

The study is continuing.

[1] This work is supported by the Office of Naval Research under contract N8onr-579 with Smith College.

REPORTS ON LALOR FELLOWSHIP RESEARCH

A new DNA fraction of the cell nucleus. JAY BARTON II.

The action of crystalline DNase on nuclei may be conveniently studied and quantitatively characterized by using isolated nuclei as the substrate. The following conclusions may be drawn: (1) Only a part of the DNA can be removed by the enzyme. (2) No protein is released with the DNA. (3) Calcium is the only other component of the nucleus released along with the DNA so far discovered.

The enzymatically resistant fraction cannot be removed by repeated digestion with fresh enzyme, by washing with water, or by washing with salt or buffer solutions of moderate ionic strength. The fraction can be removed as a complex of nucleic acid and protein by strong salt solution, 1 or 2 *M* NaCl. The complex removed in this way is not dissociated by saturated ammonium sulfate, retaining constant properties through repeated precipitations and washings and resolutions.

The amount of the resistant fraction in various species is as follows: Beef thymus lymphocyte nuclei, 77%; carp sperm, 82%; Asterias sperm, 74%; Pecten, 89%; Mytilus sperm, 84%; Arbacia sperm, 74%; turtle (Caretta) erythrocyte nuclei, 80%; Mactra (= Spisula) sperm, 40%. In all species except Mactra the basic protein of the nucleus is of the histone type. In Mactra the basic protein more closely resembles a protamine.

It is postulated that the resistant DNA is part of a complex of nucleic acid and protein held together by bonds other than electrostatic, probably co-valent.

Some exploratory experiments were performed testing the completeness of extraction of nucleoprotein from sperm by the classic procedure using 1 or 2 *M* NaCl. Tentatively, the results indicate that in those species having a small enzymatically resistant fraction, a small fraction resistant to NaCl extraction may also be discovered. In Mactra the indications are that there is a rather large fraction of DNA resistant to salt extraction. The identity of the latter fraction to the enzymatically resistant fraction has not yet been established.

Carbonic anhydrase and calcification in the fiddler crab (Uca). REINHOLD BENESCH AND RUTH E. BENESCH.

This work was designed to test the hypothesis that carbonic anhydrase functions in the formation of the crustacean exoskeleton. Molting was induced in the fiddler crab (Uca) by ablation of the eye stalks. Immediately upon completion of ecdysis the animals were placed in sea water containing varying concentrations of sulfanilamide, a powerful carbonic anhydrase inhibitor. After varying lengths of time treated animals and controls were dried for five hours at 90° C., weighed and ashed. Ca plus Mg was estimated by the Versenate method. The following observations were made: (1) The crabs were found to be more resistant to the drug than vertebrates, since they easily tolerated up to 50 mg.% of sulfanilamide for five days. (2) The Ca plus Mg content of 20 sulfanilamide-treated crabs was compared with that of 9 controls five days after molting. The average values were 8.3% for the treated and 10.2% for the control group. This difference is not statistically significant. There was, however, a striking inverse correlation between the size of the animal and its rate of calcification. (3) In a short term experiment, in which the animals were kept in 50 mg.% sulfanilamide for 24 hours after molting, the mean calcium content of five treated animals was 4.4% and that of three controls was 6.3%. The standard error of the difference between these means was 0.9 which makes this difference just significant. These preliminary results would indicate that carbonic anhydrase might play a part in the formation of the crustacean exoskeleton, but they require confirmation with a larger number of animals.

Studies on the nonsaponifiable fraction of lipid material from marine animals. LEON S. CIERESZKO.

Except for the observation by Bergmann *et al.* that cholesterol is the principal sterol of the tunicate, *Styela plicata*, nothing appears to be known about the nonsaponifiable matter of Protochordata. To extend our information along this line, two other tunicates, *Amaroucium constellatum*, and *Molgula manhattensis*, were worked up for nonsaponifiable matter. Wet *Amaroucium* yielded 0.4–0.5% total lipid. Of this 16% was nonsaponifiable. About 25% of the nonsaponifiable matter was obtained as crude sterol. Molgula, stripped of tests, yielded 0.08% nonsaponifiable matter. The sterols of the tunicates were isolated in crude form but their identification was not completed.

It was found that the pyloric caeca of the starfish, *Asterias forbesi* are rich in lipid including phospholipid. The nonsaponifiable matter of starfish caeca is rich in glyceryl ethers (about 60%), while no glyceryl ether could be detected in the lipids of starfish skin.

Urea adduct formation was applied to the fractionation of nonsaponifiable matter. Pure glyceryl ether fractions were isolated via their urea adducts from the liver oil of the shark, *Galeocerdo arcticus*, and from the fat of pyloric caeca of Asterias.

The tunicate, Botryllus, was found to contain a water soluble pigment which gave a cherry red color with concentrated sulfuric acid.

Oils of a number of marine animals, as well as aqueous extracts of Molgula and Amaroucium were prepared for later study.

Enzymes of a ribose-forming pathway during the early embryonic development of Chaetopterus. SEYMOUR S. COHEN.

A number of marine forms have been found to contain dehydrogenases essential to the oxidative formation of pentose phosphate from glucose-6-phosphate, *i.e.*, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The reduction of the coenzyme, TPN, occurs in each enzymatic reaction and the absorption of reduced coenzyme is used in the assay of the enzyme in homogenates.

It appeared of interest to determine the course of synthesis of these important enzymes in the early embryonic development of the polychaete worm, Chaetopterus, as well as the formation of ribose nucleic acid (RNA) in the embryo. An unfertilized egg contained about 2.6×10^{-6} micromoles of RNA-ribose and enough of each dehydrogenase to generate this amount of ribose in ca. 10 hours at 25°. In a 12 hour period at 22–23° in which the fertilized ovum segmented and differentiated to a ciliated swimming form of over 100 cells, there was no significant change in the RNA content of the embryo nor in the amounts of the two dehydrogenases.

Cations were observed to activate each enzyme. Mg^{++} and Ca^{++} were equivalent and optimal at .033 *M*, activating to twice the extent of Na^{+}, K^{+}, or Li^{+} which were optimal at 0.2 *M*. Concentrations higher than the optima were markedly inhibitory.

In the assay, embryos were homogenized at 0° in H_2O or 0.2 *M* NaCl and sedimented, the supernatant fluid containing the activities. Aliquots of 0.1 cc. were added to a mixture containing 1.0 micromoles substrate, cation at optimal concentration, and 0.025 *M* glycyl-glycine buffer at pH 7.4. The reaction was begun by the addition of 0.1 micromole of TPN in a final volume of 3.0 cc. It was found that the initial rate of reaction was proportional to enzyme concentration. The optimal pH range extended from pH 7.2 to 8.2.

Cyto-embryology in sea urchins. KATSUMA DAN.

The movement of the cortex of the eggs of *Arbacia punctulata* in Ca-free medium is followed by the shift of the pigment granules embedded in the cortical layer. In a dividing cell, the cortex of the cleavage furrow acquires a deep pink color while the color on the rest of the surface fades, showing the thickening of the furrow cortex. The pigment accumulation remains for a few minutes after the cleavage, then soon dissipates, so that during the resting stage, the furrow region becomes almost colorless while the polar surface assumes a deep pink color. Before the second cleavage, the pigment becomes uniformly distributed again. Since similar movements are repeated through the next cleavage cycle, at the interkinesis of the four-cell stage the cortex moves out of the interior of the larva and comes to lie on the periphery.

In sea water, the egg surface is normally bound to the overlying hyaline layer by the attachment fibers. Consequently, the cortex always remains on the periphery of the larva. The fact that the unrestricted cortical movement in Ca-free medium can give rise to the same distribution of the cortical material as that in sea water seems to be very significant from an embryological standpoint.

The tendency for the granules to return to a uniform distribution before each division must be connected with the division activity, and this accounts for the rounding-up of the blastomeres at this period.

The behavior of the hyaline layer after the mesenchyme cells free themselves from it is followed in Arbacia and Echinarachnius.

Auxin control of rhizoid-formation in Bryopsis. W. P. JACOBS.

The experiments of 1950 which verified the hypothesis that auxin controls rhizoid-formation in Bryopsis, a coenocytic alga, were confirmed and extended. Turning Bryopsis upside down and burying the shoot-tip in sand causes the buried tips to continue their growth as rhizoids; if the light intensity is high enough, the former rhizoid-forming bases will produce positively phototropic shoots. This reversal of polarity is usually preceded by migration of chloroplasts out of the buried portion and into the lighted portion. If the stem is ligated below the region of highest concentration of chloroplasts and before this migration of chloroplasts occurs, then regeneration in the region of the ligation follows the original polarity of the plant (*i.e.*, a shoot regenerates just above the ligation, a rhizoid usually regenerates just below it). These experiments do not support the idea that migration of chloroplasts is a necessary prerequisite for reversal of polarity.

Rates of feeding and oxygen uptake in some filter feeding marine invertebrates. C. BARKER JØRGENSEN.

As part of a comparative study of feeding in filter feeders, feeding rates were measured in the oyster, *Ostrea virginica*, and two ascidians, *Ciona intestinalis* and *Molgula manhattensis*. Feeding rates were computed from the rates in which the experimental animals removed finely dispersed graphite (particle size about 1 to 4 μ) from a suspension of known volume. Graphite concentrations were measured by means of a photoelectric colorimeter. In order to make possible a comparison of feeding rates in different filter feeders, the volumes of water filtered were expressed relative to the simultaneous oxygen consumption.

The rate of feeding was approximately the same in the three species. In the oyster 12–24 liters of water were filtered for each ml. of oxygen uptake. In Ciona and Molgula the figures were 8–20 liters and 10–20 liters, respectively. These values are of the same order as filtering rates previously measured in *Mytilus edulis*, *Pecten latiauratus*, and *Ciona intestinalis* (unpublished).

The ascidians retained graphite effectively only when the gill basket was covered by a sheet of mucus, secreted by the endostyle. When such mucus was present no graphite passed the filtering organs. The smallest particles were less than 1 μ in diameter. Previously it has been found that some proteins, like hemocyanin and hemoglobin, easily pass through the mucus sheet (unpublished). In the oyster graphite particles larger than about 2–3 μ were apparently completely retained by the gills whereas smaller graphite particles were able to pass through the gill filters.

The metabolism of α-glyceryl ethers in marine animals. M. L. KARNOVSKY.

The lipids of marine animals are notable for their content of α-glyceryl ethers, but although many reports of the isolation of these substances have appeared, there is no literature on their metabolism.

A rapid, sensitive method for determining α-glyceryl ethers colorimetrically has been worked out. The method depends on the specific oxidation of the glyceryl ether with periodate, and determination of the formaldehyde generated. The lipids of several species were analyzed, and Woods Hole species were found little different from their South Atlantic counterparts.

The metabolic problem was approached from three angles:

1) A possible enzymatic hydrolytic split of the ether link, with liberation of long-chain fatty alcohols, was postulated. Such enzymes have been sought, using a variety of water-soluble ether substrates, but without success.

2) The possibility of the reversible reduction of the ester link to an ether link at the α-position of the triglyceride has been examined, by using starfish digestive glands incubated aerobically or anaerobically. Highly oxygenated conditions appear to reduce the titer of α-glycerol ethers.

3) The turnover rate of the glyceryl ether fraction could be compared with that of triglyceride and phosphatide, by the use of C^{-14}-labelled glycerol. The experiments are not completed.

The possibility of the existence of a phospholipid analogous instructure to the α-glyceryl ethers has been investigated. Suggestive evidence for this has been obtained. In summary: A petroleum ether-soluble, organic phosphorus-containing substance can be isolated from the saponified phosphatide fraction of the liver lipid of the tiger shark (*Galeocerdo arcticus*). This fraction resists further alkaline hydrolysis, is precipitable by acetone from petroleum ether, is soluble in dilute aqueous alkali, and is split by an alkaline phosphatase. Ethyl ether then extracts from the very alkaline medium a long-chain α-glycol, which can be demonstrated by the ordinary α-glyceryl ether assay.

Iodine studies in Ascophyllum using radioactive iodine. SALLY KELLY AND NORMAN BAILY.

On the basis of studies using radioactive iodine, segments of the brown alga, *Ascophyllum nodosum* (Linn.) LeJolis, were found to remove iodine from sea water according to an exchange process. Over a 24-hour period the segments removed iodine at a rapid rate initially, followed by removal at a much slower rate.

The initial rapid removal is interpreted as being due to exchange between iodine in the intercellular spaces and the subsequnt slower removal to exchange between iodine in the cells. That exchange and incorporation into the cells is involved was indicated from experiments using 1) inert solutions, 2) solutions containing various amounts of iodides, 3) hourly replenishment with fresh solutions.

By varying the concentration of iodides in the surrounding medium from 200 to 5000 gamma per liter, it was observed that the concentration of iodine within the segments appeared to be independent of the concentration outside. The concentration of iodine inside the segments could be calculated from the amount of radioactive iodine exchanged, and preliminary calculation indicates an internal concentration of approximately 0.2 mg./g. dry weight. This represents a relative concentration of iodine within the algae about 200 times that of an equal volume of water.

Iodine was found to be taken up in the form of iodides and not as iodates, although the latter is thought to be present in greater concentration in sea water.

That the exchange is characteristic of living protoplasm was demonstrated by the fact that there was no removal of radioactive iodine from solution by dead segments.

The valence of copper in hemocyanin. MARTIN P. SCHULMAN AND GEORGE WALD.

It is commonly believed that in hemocyanin, as in hemoglobin, the metal ion remains reduced on oxygenation. Yet, viewed without prejudice, many properties of oxyhemocyanin agree better with the idea that it is a cupric complex. It is blue, a cupric color; and its absorption band at about 575 mμ lies close to those of artificial cupri-protein complexes. The "methemocyanin" supposedly produced by treatment with strong oxidants retains essential properties of hemocyanin itself, including reversible combination with oxygen. The compound allegedly formed by hemocyanin with CO has the spectrum simply of reduced hemocyanin. It might readily be supposed therefore that the combination of hemocyanin with oxygen is a true oxidation, and that in oxyhemocyanin, copper is in the cupric state.

This would make hemocyanin analogous to a copper oxidase. We have inquired into its oxidase activity. Defibrinated Limulus blood oxidizes cysteine, ascorbic acid and catechol with molecular oxygen; tyrosine is not oxidized. This activity, however, resides almost entirely in the serum from which all hemocyanin has been ultracentrifuged. It appears to be due there to

catalysis by traces of heavy metal. Hemocyanin itself, purified by ultracentrifugation and washing, has almost negligible oxidase activity with these substrates.

Defibrinated Limulus blood reduces itself, turning colorless on standing. It displays also a measurable oxygen uptake. Purified hemocyanin, however, neither reduces itself nor consumes oxygen. It does both on addition of blood ultracentrifugate or a boiled extract prepared from it.

It is well known that proteins generally yield a violet coloration on treatment with cupric ions and dilute alkali. We have found—and then learned that it has been known since Henze (1901)—that oxyhemocyanin yields this test on simply adding alkali; the hemocyanin itself provides both the protein and the cupric ions. We find, however, that no coloration is obtained on adding alkali to reduced hemocyanin (Limulus). This seems to indicate that copper is cupric in oxyhemocyanin, and cuprous in reduced hemocyanin.